REGULATION OF *C. ELEGANS* SPERM ACTIVATION BY THE PROTEASE INHIBITOR SWM-1

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

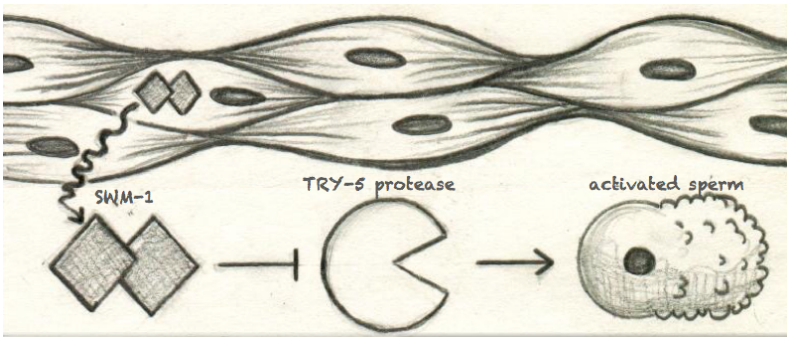
Creation of the next generation by sexual reproduction requires the fusion of male and female gametes. For many species, this entails a sessile oocyte and a highly motile spermatozoon with remarkable ability to sense and migrate through the often structurally and chemically complex reproductive tract of a female. Despite many years of research, our understanding of the basic components of male-factor infertility, in any species, is poor. While we know of many factors involved or associated with sperm success, few have been well characterized at the molecular level. Understanding how sperm successfully accomplish their journey is critical to human health and the health of ecosystems that hinge on the reproductive success and balance of species within.

The study of reproductive biology is challenging partially due to the lack of direct conservation in many genes involved in the process of reproduction. Therefore, it is critical to investigate reproduction in a wide variety of species both to identify conserved themes and also to discover novel cell biological processes.

In this dissertation, I study the secreted serine protease inhibitor SWM-1, a protein known for its critical role in male fertility of the nematode *Caenorhabditis elegans*. Through this work, I have discovered that SWM-1 is produced in an unlikely source of somatic cells: body wall muscle. From muscle, SWM-1 is secreted into the body fluid of the worm and enters the gonad to perform its critical role of preventing sperm from premature motility, which causes male infertility. I show that there is bi-

directional movement of proteins between the body fluid and gonadal structures, which may be a widespread phenomenon. Through this work, I also discovered that SWM-1 is a seminal fluid protein and a component of the sperm migratory environment though its role in this environment remains unknown. I expand on our knowledge of the molecular relationship to SWM-1's likely target, the protease TRY-5, by probing its protease inhibitory domains and visualizing SWM-1 and TRY-5 in combination. Lastly, I began initial characterization of other mutants that affect the acquisition of motility.

This work adds to the knowledge of the role of proteolysis regulators in male reproductive success and the regulation of cell motility. Moreover, my finding that muscle is a source of SWM-1 and that SWM-1 is also a seminal fluid protein reveals a novel means of ensuring sperm success and highlights the utility of *C. elegans* as a system to study secretory and reproductive biology.



Protease signaling regulates sperm activation in C. elegans

Artwork by Miranda Lavallee

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CHAPTER 1

INTRODUCTION

Sexual reproduction requires the generation of highly differentiated sperm cells with specialized structures and cellular characteristics that support navigation to and fusion with an oocyte. However, immediately following meiosis, sperm can neither migrate, nor fuse with an oocyte. To gain fertilizing ability, sperm morphology and physiology transform significantly to permit movement, navigation, and fusion. This transformation is regulated exclusively by cues that originate from the male and female reproductive tracts, as the tightly compacted genome of the sperm cell does not synthesize new protein products to aid in the demands of its journey (Figure 1.1) (reviewed in Gervasi and Jin and Yang, 2017 and Visconti, 2017).

Studies on postmeiotic sperm development have been carried out in a variety of model and nonmodel organisms as well as in humans, revealing interesting differences and similarities between species. One significant difference is at the genetic level: genes that regulate sperm maturation are some of the most rapidly evolving genes in the genome (Swanson and Vacquier, 2002). Sperm morphology is also highly variable between species (Alvarez, 2017). Despite these genetic and morphological differences, the classes of proteins involved in reproduction are conserved in many taxa and morphologically diverse sperm undergo analogous maturation processes, many of which

are carried out by conserved cellular machinery (Figure 1.1) (Fraire-Zamora and Cardullo 2010; Mueller et al., 2004). Thus, a better understanding of sperm maturation from the perspective of a wide variety of species will aid in our knowledge of both conserved and novel reproductive and cell biology.

The study of sperm development in a wide range of species has also uncovered interesting secretory biology and highly regulated production of sperm maturation cues by many cell types along the length of winding, tube-shaped reproductive tracts. Overall, this process is poorly understood but provides an opportunity to understand how an organism can create a complex signaling environment using inputs from many different cell types. Furthermore, although many mammalian reproductive systems are studied extensively, including mouse, bull, boar, and stallion, many suffer from a lack of testable *in vivo* or *ex vivo* models. Thus, investigation of sperm maturation in a variety of species is important for understanding male-factor infertility, mechanisms of cell migration, cell secretion, and evolution through sexual selection.

This is a dissertation on the regulation of postmeiotic sperm maturation in the nematode *Caenorhabditis elegans.* In this male/hermaphrodite species, the sperm maturation process is known as sperm activation and is regulated by proteolysis regulators present in the male and hermaphrodite reproductive tracts. The worm has proven to be an excellent model system to study various of aspects of reproductive biology including the germline stem cell niche, molecular mechanisms of meiosis, oocyte development and postmeiotic sperm maturation (Figure 1.1, Figure 1.2) (Corsi et al., 2015). In this introduction, I briefly describe important aspects of postmeiotic sperm maturation in the male and female reproductive tracts, drawing from our knowledge in

various mammals, insects and *C. elegans*, while highlighting the role of proteolysis regulators that are important for sperm function and cell motility.

Sperm maturation in the male reproductive tract

After meiosis, sperm produced in the testis enter the somatic gonad, tube-shaped tissues through which they pass and begin the maturation process prior to being transferred to a female. In mammals, these tissues include the epididymis and the vas deferens. Insects and *C. elegans* have simplified male reproductive tracts but many parallels can be made, as they are also tube-shaped structures that secrete complex repertoires of proteins from a variety of different cell types (L'Hernault, 2017; McGraw et al., 2015; Nishimura). In mammals, the male reproductive tract causes changes to sperm cell physiology that prime the cell for its journey in the female. These regulated changes include significant alterations to the molecular composition and morphology of the sperm membrane and intracellular physiology that allows sperm to progressively gain motility (Figure 1.3) (Dacheux et al., 2003). Our current understanding of the sperm maturation processes that takes place in the male reproductive largely relies on comparisons between sperm and somatic gonad tissue analyzed prior to, during, and after transit through the epididymis and vas deferens as well as immunohistochemical analysis and gene expression profiling of different parts of the reproductive tract (Belleannée et al., 2012; Chalmel and Rolland, 2015; Zhang et al., 2006). While these studies have identified many factors that are important for sperm development, few of these factors have been experimentally verified. Thus, understanding the molecular mechanisms that will be helpful for developing fertility or contraceptive treatments will benefit from the

development of *in vitro* and *ex vivo* models, and model organism studies, as many maturation factors have conserved molecular structure and function (Druart et al., 2013; McGraw et al., 2015).

Changes in sperm membrane composition

During epididymal maturation, the membrane composition of sperm is significantly altered to prepare the sperm to survive in the female. As one mechanism for this, the sperm membrane can be altered by the incorporation of proteolysis regulators that have important functions in the later part of the journey in the female. For example, the serine protease inhibitor Spink-3 (serine protease inhibitor kazal-type 3), is produced in the seminal vesicles of mice and incorporated into the sperm membrane during transit through the epididymis (Lai et al., 1991; Ou et al., 2012). This protease inhibitor is transferred with sperm to females during mating and is progressively removed from the membrane during migration through the female; whereas as uterine sperm have Spink-3 in their membranes, sperm that have migrated to the oviduct lack Spink-3 (Ou et al., 2012). Although the exact function(s) of Spink-3 are not precisely defined, it is proposed to inhibit female-derived proteases and prevent premature acrosome reaction until the sperm cell reaches the oocyte (Ou et al., 2012; Zalazar et al., 2012).

Another important change that occurs is alterations of the sperm gylcocalyx, the coat of sugar molecules in the sperm membrane. As sperm enter the epididymis, the surface of their membrane becomes decorated with various sugar molecules acquired from different parts of the epididymis. In humans, some glycoproteins that sperm acquire during transit through the epididymis include GPI-anchored proteins that are important

for immune protection, and beta defensin, which is important for navigating the female reproductive tract (Kirchhoff and Hale, 1996; Tecle and Gagneux, 2015). These proteins are secreted from the epithelium of the epididymis and can be incorporated into the sperm membrane. Furthermore, humans express lectins, a class of carbohydrate-binding proteins along the length of the epididymis, which are expressed in region-specific patterns, suggesting that regulation of sugars on the surface of the sperm membrane is important (Arenas et al., 1996). The lipid content of the sperm membrane is also altered during epididymal transit. Analysis of sperm prior to and following epididymal maturation has shown that the cholesterol content is decreased in more mature sperm (Gervasi and Visconti, 2017).

Not only can the sperm membrane be altered by secretions in the lumen of the epididymis, sperm can acquire proteins and gain membrane from exosomes secreted by the reproductive tract known as epididymosomes (Cooper, 1998; Martin-DeLeon, 2015). Roles for epididymosomes have been implicated in preparing the sperm membrane for survival, motility, and fusion (Girouard et al., 2011; Sullivan et al., 2005; Sullivan et al., 2007). Epididymosomes carry a wide variety of cargo that differs depending on the location along the epididymis from which they are isolated. For example, proteomic analysis of epididymosomes purified from bovine vas deferens showed that its cargo includes proteases and protease inhibitors (Girouard et al., 2011). These types of studies are challenging to perform in humans yet one study has analyzed the proteome of human epididymosomes of men undergoing vasectomy reversal. This study revealed that purified epididymosomes contain proteolysis regulators, some of which previously have been shown to have important sperm maturation roles (Thimon et al., 2008). Recent

work has provided evidence that epididymosomes can also deliver RNA molecules, which has been proposed to have implications in modifying DNA methylation (Reilly et al., 2016).

Exosomes have also been reported in the male reproductive tract of *Drosophila*. In this study, researchers showed dynamic fusion events of exosomes *in vivo*, an important step in understanding the role and molecular mechanisms by which epididymosomes may fuse with sperm (Corrigan et al., 2014). In *C. elegans*, such vesicles secreted by the male have not been reported. However, larger seminal fluid-containing vesicles are present in the *C. elegans* vas deferens. Furthermore, membrane from these vesicles is transferred to hermaphrodites during mating (Angela Snow and Gillian Stanfield, University of Utah, personal communication). Although the presence of epididymosomes in *C. elegans* is unproven, this system would provide an excellent opportunity to investigate membrane dynamics as the worm is transparent. Overall, it is well accepted that epididymosomes play a role in sperm maturation in many systems, but much more investigation is needed to determine the mechanism of cargo transfer.

In flagellated sperm, the most obvious change to sperm cell morphology during maturation in the male includes migration of the cytoplasmic droplet (CD). During meiosis, sperm release a residual body of cytoplasm with the exception of the CD, which moves posteriorly after transit through the epididymis. The role of the CD is not clear although it likely provides many maturation factors and regulates several processes as a variety of molecules have been identified in purified CDs. For example, the metalloprotease ADAM3 is required for sperm to enter the female, a process that is dependent on the CD protein Tex10 (Gervasi and Visconti, 2017; Yamaguchi et al.,

2009).

Sperm maturation in the female reproductive tract: seminal fluid and female response

Once sperm leave the male and enter the female, they encounter a tremendously complex environment. In the female, sperm face the challenge of adapting to pH, hyperactivating flagellar beating, avoiding the female immune response, navigating to the oocyte, and competing with other sperm. Lastly, sperm must undergo the acrosome reaction, a specialized exocytic event that allows fusion with the egg. Combating these challenges requires inputs from the female and also from males in the form of seminal fluid. Collectively, the sperm maturation processes that take place in the female, which results in a fertilization-competent cell, is known as capacitation.

Seminal fluid composition and function

The majority of semen is made up of nonsperm components. Seminal fluid includes proteins, carbohydrates, lipids, and some immune cells (Poiani, 2006). The molecules in seminal fluid have critical and diverse functions in fertility including: facilitating and regulating sperm migration, navigation, completion of the acrosome reaction, and penetration of the oocyte oolemma. However, for the large majority of seminal fluid factors, functions have not been determined (Robertson and Sharkey, 2016; Wolfner, 2007). Various studies have sought to identify the components of seminal fluid in humans, agriculturally important bovines, and various insects (Druart et al., 2013; Jodar et al., 2017; Mueller et al., 2004; Pilch and Mann, 2006; Robertson, 2007). These studies have revealed that there are a large number of proteins in seminal fluid. For

example, there are ~900 in humans and ~200 in *Drosophila* (Laflamme and Wolfner, 2013). While most of the seminal fluid proteins that have been identified are not conserved between species, the major classes of proteins represented are conserved among evolutionarily distant taxa (Druart et al., 2013).

Classes of conserved proteins include proteases and protease inhibitors, mucins, carbohydrate-binding lectins and insulins (Pilch and Mann, 2006; Poiani, 2006). In humans, proteolysis regulators compose ~10% of the proteins in seminal fluid (Laflamme and Wolfner, 2013). One well-known role for seminal fluid proteins is to aid in the regulation of sperm migration. In mammals, sperm progressively begin moving their flagella during transit in the epididymis. However, in the female, seminal fluid forms a coagulum primarily formed of semenogelin proteins, which forms a clot that restricts sperm movement (Jonsson et al., 2005). Within minutes, a chymotrypsin-like protease, prostate-specific antigen (PSA) degrades semelogelins, freeing sperm to continue to the oocyte (de Lamirande, 2007). Another mammalian proteolysis regulator important in the capacitation process is the serine protease inhibitor, Kasal-type-like protein (SPINKL). SPINKL is expressed exclusively in the seminal fluid-producing seminal vesicles and transferred to the female during mating. *In vitro*, SPINKL inhibits capacitation but its role *in vivo* has not been determined (Lin et al., 2008).

Perhaps the most thoroughly investigated organisms in terms of seminal fluid composition, production, and function, is *Drosophila.* In the fly, there is a cascade of proteolytic events that begins in the male with cleavage of the trypsin-like protease, called seminase (Laflamme et al., 2012). This proteolytic cleavage is important for the binding of a peptide hormone, sex peptide, to sperm, which in turn is responsible for a

collection of events known as the female mating response. The female mating response includes increased egg laying and release of stored sperm (Chapman et al., 2003). As in mammals, *Drosophila* male reproductive tract tissues secrete two carbohydrate binding lectins. These lectins are produced in the male accessory glands and are components of seminal fluid required for the female mating response (Sitnik et al., 2016). Indeed, the fly has been a critical model for studying reproduction due to its anatomically analogous tissues and conserved molecular machinery (Wolfner, 2002).

Some seminal fluid proteins cause significant morphological changes required for regulation of motility, especially in the case of aflagellate sperm. Some species that produce aflagellate sperm include nematodes (round worms), Annelids (segmented worms), and some species of Arachnids (spiders) (Morrow, 2004). The most well studied aflagellate sperm are those of the nematodes, *C. elegans* and *Ascaris,* which undergo a dramatic transformation called sperm activation in response to proteolysis regulators derived from the male reproductive tract (Figure 1.2). During sperm activation, the cell membrane is rearranged and becomes polarized with a cell body that contains the nucleus at one end and a pseudopod, which is used to crawl, at the opposite end of the cell (Figure 1.2) (Bottino et al., 2002; Shakes and ward, 1989; Ward et al., 1983). This process is triggered by the trypsin-like protease, TRY-5, a serine protease that is a component of seminal fluid. Sperm activation *via* TRY-5 activity is restricted in the male by a protease inhibitor, SWM-1, expressed in both males and hermaphrodites (Chapter 2). Once TRY-5 is transferred to hermaphrodites in seminal fluid, sperm become activated in the uterus and begin crawling towards oocytes (Smith and Stanfield, 2011; Stanfield and Villeneuve, 2006). Interestingly, SWM-1 is also a seminal fluid protein, and it is

transferred to hermaphrodites along with TRY-5 (Chapter 2). While SWM-1 in the hermaphrodite can affect male fertility, how and why it affects sperm in the uterus remains elusive (Chapter 2 and Appendix). While there is evidence that SWM-1 directly inhibits TRY-5, the exact molecular mechanism by which they act to regulate activation has yet to be determined.

Another known seminal fluid protein in *C. elegans* is the mucin PLG-1 (Hodgkin and Doniach, 1997; Palopoli et al., 2008). PLG-1 forms a mating plug in the hermaphrodite vulva following copulation to prevent mating by other males. Aside from PLG-1, TRY-5 and SWM-1, no other seminal fluid proteins have been confirmed in *C. elegans*. However, there are a number of putative seminal fluid proteins that are expressed in the male vas deferens and represent the classes of seminal fluid proteins commonly found in most species. These include an insulin, another mucin, and a lectin (Angela Snow, Gillian Stanfield, University of Utah, personal communication; Thoemke et al., 2005). Although few seminal fluid proteins are well-characterized in *C. elegans*, collectively, seminal fluid has been shown to have a dramatic effect on the health of the hermaphrodite after mating, causing shortened life span in an insulin signaling-dependent manner (Shi and Murphy, 2014).

Changes to the sperm membrane in the female

The central event of fertilization is the fusion of the two gamete membranes. Thus, the sperm cell is designed to precisely sense its target and prepare for fusion. The molecular means by which sperm prepare for this fusion is challenging to study *in vivo,* and most studies have been performed *in vitro,* though *in vivo* mouse models are

increasingly being utilized. While these mechanisms are not well understood, it is well accepted that changes in membrane fluidity *via* reduction of cholesterol and controlled production of reactive oxygen species is important (reviewed in De Jonge, 2017; de Lamirande et al., 1997; Stival et al., 2016;). Furthermore, until sperm are in close proximity to the egg, the flagellar beating is not sufficient to penetrate the egg oolema. Rather, sperm must initiate what is called hyperactivation of flagella, an asymmetrical, rapid flagellar beating motion which is regulated by an influx of calcium through cation channel sperm-associated (CatSper) channels (Suarez, 2008).

In the reproductive tract of mammalian females, the most significant membrane rearrangement is the acrosome reaction. The acrosome is a specialized sperm organelle located in the sperm head which fuses with the sperm membrane during a highly-regulated exocytic event (Ickowicz et al., 2012). Only an acrosome-reacted sperm cell can penetrate the oocyte and complete successful fertilization. Furthermore, spatial and temporal regulation of the acrosome reaction is critical as fertilization requires that it occur only when sperm is in close proximity to the oocyte in the oviduct (De Jonge, 2017). *C. elegans* sperm also perform specialized membrane fusion events required for fertilization. In *C. elegans*, sperm contain multiple golgi-derived membranous organelles (MOs) that fuse with the plasma membrane, which is analogous to the acrosome reaction and prerequisite for fertilization. MOs are highly regulated to release major sperm protein, which acts as both a motility-driving protein in the pseudopod and also a signaling molecule released from sperm in the uterus (Fraire-Zamora and Cardullo, 2010).

Protease signaling in the regulation of cell motility

Aside from sperm, many other cell types are required to migrate, for example, as a mechanism of wound healing and also during developmental processes such as gastrulation (Heutinck et al., 2010; Rau et al., 2007) (Keller, 2005). Regulation of cell migration *via* proteolysis is an efficient means to create a rapid, posttranslational response such as those needed during immune response to injury and the acquisition of sperm motility. On the other hand, misregulation of cell migration can lead to various disease states including cancer metastasis, which involves proteolytic degradation of the extracellular matrix (Stetler-Stevenson et al., 1993). Other cell motility-regulating mechanisms are required intracellularly. Such is the case for SERPINB1, an intracellular protease inhibitor that regulates proteolysis in response to cellular stress. When misregulated, SERPINB1 has been associated with oral cancers and with increased cell migration upon overexpression (Torriglia et al., 2017).

Dissertation summary

The cellular mechanisms by which sperm success is ensured are undoubtedly complex and challenging to study. Considering the intricacy of the tissues that generate sperm maturation cues, and the extensive cell signaling involved, it is not surprising that our current understanding of male-factor infertility, in any organism, is far from complete. Yet given our rapidly changing environment, increasing age of paternity, and recently compiled large-scale data showing a severe decline in human sperm concentration, it is important to gain a basic understanding of what makes sperm successful. Since reproductive-related genes are rapidly evolving, it is critical to carry

out studies in a wide variety of organisms (Levine, 2017).

In this dissertation, I study the role of the protease inhibitor SWM-1 in the regulation of *C. elegans* sperm activation. In Chapter 2, I use precise genetic manipulation and microscopy to analyze the expression and protein localization of SWM-1. Using tissue-specific expression of SWM-1, I find that it is produced in, and secreted from, extra-gonadal muscle cells into the body cavity of the animal, where it is present in the body fluid from which it can enter the gonad and inhibit premature activation by the protease TRY-5. I expand these studies to test models of inhibition of TRY-5 and also explore the extent to which other molecules are exchanged between body fluid and the gonad.

In Chapter 3, I describe my work testing the role of the SWM-1 protease inhibitor domains. These results provide additional evidence supporting previous results, which suggested that SWM-1 may have multiple protease targets. In Chapter 4, I describe preliminary analysis of the results of a *swm-1* suppressor screen that sought to identify additional genes involved in the regulation of sperm activation. Lastly, the Appendix contains a preliminary analysis of the role of *swm-1* in sperm competition. I conclude with a brief summary of my findings, extended interpretations of results, considerations for future work on these projects, and I share my ideas on how to test the many new questions that have arisen from my findings.

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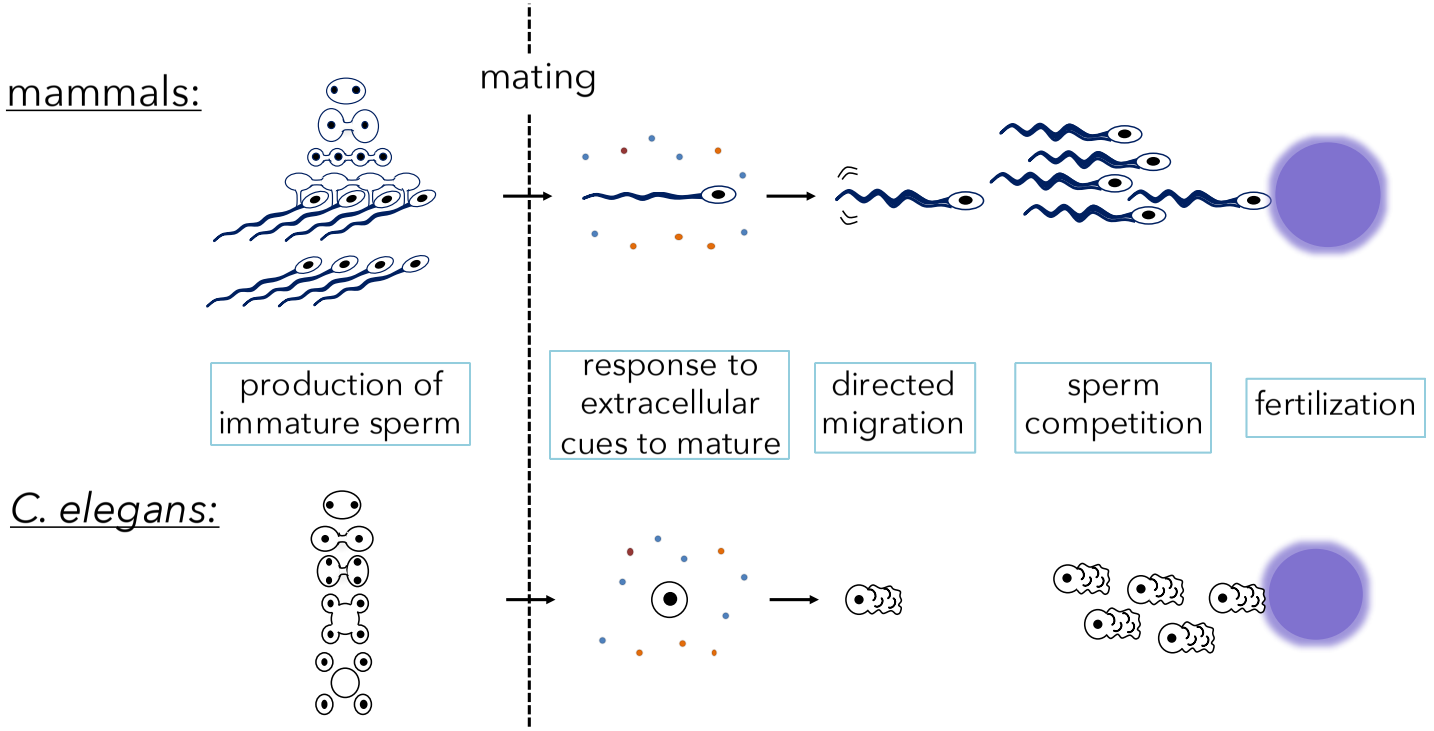
Figure 1.1 C. *elegans* sperm use conserved cellular machinery. Schematic of postmeiotic sperm maturation in mammals and *C. elegans.* The product of mammalian and *C. elegans* male meiosis is an immature sperm cell that is not competent to fertilize an oocyte. Both sperm are transcriptionally quiescent and rely on extracellular maturation cues to undergo directed migration and specialized membrane changes including exocytic events to be fertilization competent. List includes *C. elegans* genes that are involved in the indicated process which are analogous or conserved between mammals and *C. elegans*.



Meiosis
puf-8 (pumilio-like)
wee-1.3 (cell division)
spo-11 (DSB formation)
msh-5 (mismatch repair)
chk-2 (checkpoint kinase)
him-8
zim-1,2,3

Organelle function
spe-4 (presenilin)
spe-5 (V-ATPase)
spe-6 (casein kinase)
spe-10 (DHHC-CRD)
spe-39 (vesicular trafficking)
fer-1 (dysferlin)
spe-17

Activation/ Spermiogenesis
spe-8 group
swm-1 (protease inhibitor)
try-5 (serine protease)

Sperm guidance
prostaglandins

Fertilization
trp-3 (calcium channel)
spe-9 (EGF repeats)
spe-42 (RING finger)
spe-45 (Izumo-like)
fer-14
spe-38

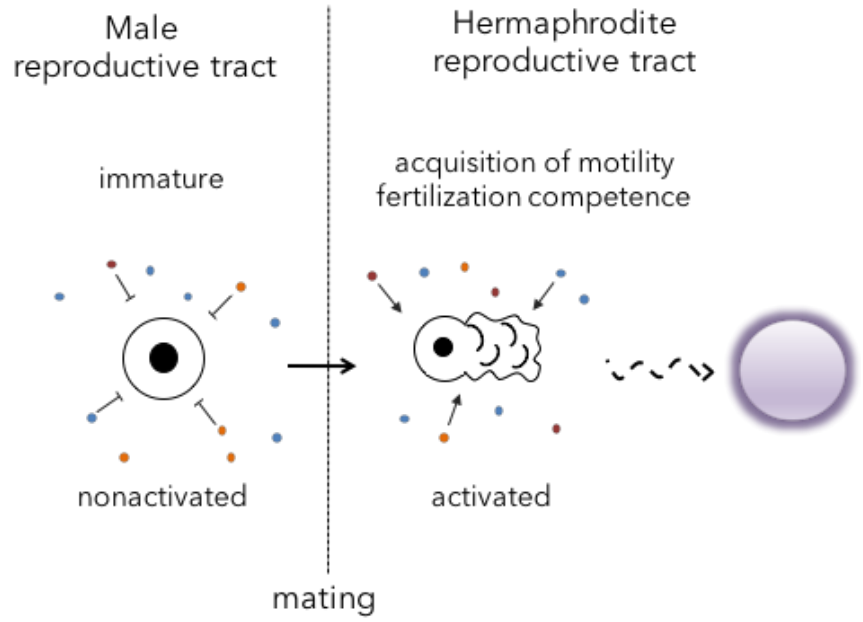


Figure 1.2 Spatial and temporal regulation of *C. elegans* sperm activation. Sperm are produced in the male reproductive tract as round, immotile spermatids and maintained in the nonactivated state until transfer to the hermaphrodite. In the hermaphrodite, sperm become polarized and extend a pseudopod used to crawl towards oocytes. Activation is regulated by extracellular signals.

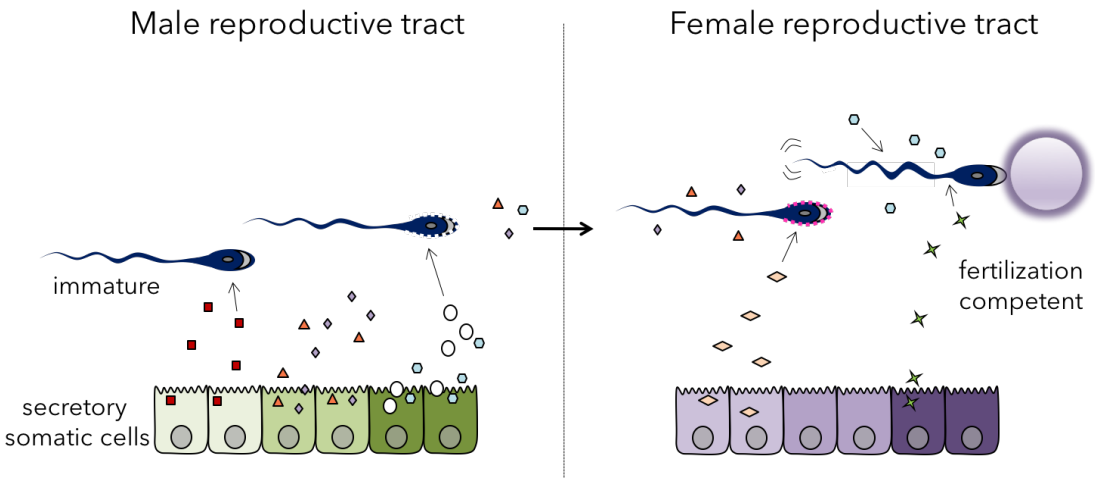


Figure 1.3 The extracellular environment regulates sperm motility. Schematic of maturation processes important for fertility. Changes to the sperm occur in response to signals secreted by the male and female reproductive tracts. The sperm membrane composition is altered in males and females to prepare sperm for fusion with the egg in response to female signals and male signals transferred in seminal fluid. Full flagellar beating and the acrosome reaction occurs in the female reproductive tract.

CHAPTER 2

THE MUSCLE-DERIVED PROTEASE INHIBITOR, *SWM-1,* REGULATES *C. ELEGANS* SPERM MOTILITY

Abstract

The development of highly differentiated sperm cells that specialize in navigating to and fusing with an oocyte is critical for sexual reproduction. As a major part of their differentiation, sperm undergo postmeiotic maturation en route to the oocyte. In *C. elegans,* this maturation process is called sperm activation, and it transforms immotile spermatids into migratory, fertilization-competent cells. Here we use precise genetic engineering to study the protease inhibitor SWM-1, the negative regulator of *C. elegans* sperm activation, to understand how males generate a signaling environment that precisely regulates activation at the level of the whole organism. We show that SWM-1 is produced in an unexpected source of extragonadal cells: body wall muscle. From muscle, SWM-1 is secreted into the body cavity and enters the gonad; there it is in close proximity with its likely target, TRY-5, a secreted serine protease that triggers activation. We extend these studies to show that the somatic gonad and body fluid can exchange other factors, which may be a means of soma-germline communication for other reproductive processes. Through genetic analysis, we determine that SWM-1 and TRY-5 can localize to the gonad independently of one another, expanding on our understanding of how these protease signals regulate the acquisition of motility. Lastly, we show that

SWM-1 is a component of the sperm migratory environment and is contributed by males in seminal fluid and also by hermaphrodites.

Introduction

Sexual reproduction requires the development and differentiation of sperm cells capable of migrating to and fusing with an oocyte. To become fertilization competent, sperm undergo significant postmeiotic morphological and physiological changes, all of which occur in the absence of synthesizing new protein products. This maturation occurs in response to extracellular cues generated by specialized support cells of the male and female reproductive tracts. In mammals, sperm progressively mature within the male as they pass along the considerable length of the male gonad. Within the labyrinthine epididymis and the vas deferens, different cell types secrete distinct repertoires of factors including proteins, sugars, lipids, and vesicles (Belleannée et al., 2012; Zhang et al., 2006). These cues cause changes to sperm membrane fluidity and cell morphology, and also trigger biochemical modifications required for fertilization (reviewed in Gervasi and Visconti, 2017). Within the female, additional male and female factors complete the maturation process required for maximum forward movement and induction of the acrosome reaction, resulting in the highly specialized cell that locates and fuses with the oocyte (reviewed in Abou-haila and Tulsiani, 2009; de Lamirande et al., 1997; and Salicioni et al., 2007).

In all species that have been investigated, a significant proportion of factors that regulate postmeiotic sperm development are secreted proteolysis regulators (Jodar et al., 2017; Laflamme and Wolfner, 2013). For example, in mice, proteolysis-regulated

processes include increased flagellar beating by degradation of proteins that inhibit motility, as well as proper localization of the sperm cell in the female reproductive tract (Christensson et al., 1990; Jonsson et al., 2005; Martin Robert et al., 1997; Yamaguchi et al., 2009) . In *Drosophila*, the seminal fluid protease inhibitor Acp62F is important for ovulation and is also involved in sperm competition (Mueller et al., 2008).

The nematode *Caenorhabditis elegans* represents a simple model to study how protease signaling regulates sperm maturation and post-translational cell motility. In *C. elegans*, both males and hermaphrodites produce amoeboid sperm (Ward and Carrel, 1979; Ward, 1977). Meiotic cell division results in haploid spermatids that are initially immotile. Upon receiving a signal, spermatids become polarized and generate pseudopods, which they use to migrate towards oocytes (Bottino et al., 2002; Ward and Carrel, 1979). This differentiation process is called sperm activation, and it is thought to result in spermatozoa that are fully competent for fertilization. Spermatids are stored in their nonactivated form within a region called the seminal vesicle. During mating, cells along the length of a tube-shaped vas deferens release seminal fluid components at specific times and locations, and these are transferred along with spermatids to the hermaphrodite uterus. Rapid activation into functional spermatozoa ensues.

Two critical regulators of sperm activation are the protease TRY-5 and the trypsin inhibitor-like (TIL) protein SWM-1 (Smith and Stanfield, 2011; Stanfield and Villeneuve, 2006). TRY-5 is thought to be the sperm activation-promoting signal in seminal fluid. Conversely, SWM-1 inhibits activation; *swm-1* mutant males contain prematurely activated sperm and are frequently infertile due to the inefficient transfer of sperm (Stanfield and Villeneuve, 2006). TRY-5 is required for this premature activation,

and it is visible at elevated levels near activated sperm in *swm-1* mutants. Thus, by their opposing activities, SWM-1 and TRY-5 ensure male fertility by signaling sperm differentiation to occur only at the right place and time. Yet how SWM-1 inhibits TRY-5 from prematurely activating sperm in the male has been unknown, both in terms of where SWM-1 originates and its relationship to TRY-5 localization and/or activity.

Here, we describe how this critical developmental transition is regulated at the whole-organism level. Using CRISPR-mediated genome engineering and analysis of protein localization in mutants defective for regulators of sperm activation, we determine where SWM-1 localizes and functions in conjunction with TRY-5. We show that, surprisingly, the SWM-1 inhibitor is produced in and secreted from extra-gonadal muscle cells into the body cavity (pseudocoelom) that surrounds the gonad. SWM-1 then enters the seminal vesicle, where it is coincident with TRY-5 protein localization. Furthermore, SWM-1 produced in the gonad is released into the body cavity, and this bidirectional exchange of proteins between the body cavity and the somatic gonad may be a general phenomenon. Lastly, we find that SWM-1 is contributed by males in seminal fluid, and also by hermaphrodites to the sperm migratory environment, the hermaphrodite uterus, where it can affect male fertility.

Results

swm-1 is expressed in the male somatic gonad and in extra-gonadal muscle cells

We sought to determine where SWM-1 is expressed in males. To identify cells that produce SWM-1, we generated strains carrying a transcriptional reporter, *Pswm-1::mCherry::H2B*, in which the *swm-1* promoter region controls expression of an

mCherry-tagged histone protein (Merritt et al., 2008). Based on its amino acid sequence, SWM-1 is predicted to be a secreted protein; this strategy allowed us to identify cells that express *swm-1* based on the position of mCherry-positive nuclei. We used Mos-mediated Single-Copy Insertion (MosSCI) to insert the desired sequences at the *ttTi5605* locus (Materials and methods and supplemental data) (Jensen et al., 2008). Importantly, control strains with this promoter driving expression of full-length SWM-1 showed strong rescue of the *swm-1(me87)* null mutation (Supplemental data).

We found that *swm-1* is transcribed in both gonadal and extragonadal somatic tissues (Figure 2.1). In *C. elegans* males, the gonad forms a tube structure with germline stem cells at the distal end that proliferate and move proximally to undergo meiotic divisions and give rise to spermatids. At the L4 larval stage of development, spermatids begin to be produced and are stored in the seminal vesicle (Kimble and Hirsh, 1979). The vas deferens is at the proximal end of the gonad and forms a complex secretory tube through which sperm pass during mating. The vas deferens contains at least three cell types: valve cells, cuboidal cells, and elongated cells (Lints and Hall, 2009), two of which have been shown to produce seminal fluid (Smith and Stanfield, 2011). By 24 hours post-L4, adult males have a fully-developed gonad and sperm have accumulated in the seminal vesicle.

We observed *Pswm-1::mCherry::H2B* reporter expression in 6-8 of the twelve vas deferens cuboidal cells (Figure 2.1A,B). We also observed *Pswm-1::mCherry::H2B* in posterior body wall muscle cells and in the male-specific diagonal muscle cells, both of which overlie the seminal vesicle and vas deferens region (Figure 2.1C-F) (Sulston et al., 1980). We confirmed the identity of these cells by co-localization with a

Pmyo::GFP::H2B reporter transgene, which drives expression of nuclear-localized GFP in body wall and diagonal muscles (Figure 2.1E,F) (Fire et al., 1990). Expression of the *Pswm-1* reporter in both gonadal and muscle cells began at late larval stages and persisted until at least 48 hr post L4 (supplemental data). Additionally, we observed reporter expression at low levels in two cells in the head, which likely were neurons (supplemental data). These data indicate that *swm-1* is not expressed in sperm. Instead, it is produced in somatic tissues, including the vas deferens, which is involved in the production and release/transfer of seminal fluid, as well as in tissues outside the gonad, muscle cells. These data suggest that somatic tissues are important for the regulation of a crucial step of sperm development, the acquisition of motility.

SWM-1 is secreted into the body cavity and surrounds sperm in the seminal vesicle

To determine where SWM-1 protein localizes, we generated worms expressing a SWM-1::mCherry fusion protein. Using a knock-in CRISPR strategy adapted from Arribere (2014) and Ward (2015), we inserted mCherry coding sequences at the endogenous *swm-1* locus. Briefly, we targeted Cas9 to two sites, one in *swm-1* and one in the neighboring gene *C25E10.8*, while providing a repair template to introduce mCherry (Materials and Methods, supplementary data). We isolated two independent lines: *swm-1(jn60)* and *swm-1(jn62)*. To determine if these strains had the desired alterations, we sequenced the *swm-1* gene and repair template region in both lines. The *jn62* allele had repaired as designed in the template. However, the *jn60* allele contained an alteration of amino acids 24 and 25 from two glutamate residues (EE) to valine and lysine (VK), as well as a silent mutation in codon 22. To test if these SWM-1::mCherry

fusion proteins were functional, we quantified sperm activation in *jn62(swm-1::mCherry*) and *jn60(swm-1(EEtoVK)::mCherry)* males (Figure 2.2A). *jn62[swm-1::mCherry]* male sperm appeared essentially wild-type, although we observed slightly elevated levels of sperm activation in older adults. Thus, SWM-1::mCherry was functional and capable of inhibiting activation *in vivo*. However, the *jn60(swm-1(EEtoVK)::mCherry)* line showed increased levels of activation in both 24 hr and 48 hr adults as compared to either the *jn62* line or wild-type males (Figure 2.2A), indicating that the introduced mutations impaired *swm-1* function. Thus, we primarily used the *jn62* line to analyze SWM-1 localization, largely focusing on localization in adult males, when regulation of activation is most critical. However, we also examined protein localization throughout development (supplementary data).

In adult males, the SWM-1::mCherry protein localized to cells where we observed the transcriptional reporter: 6-8 of the twelve vas deferens cuboidal cells, diagonal muscles, and body wall muscles (Figure 2.2B-E). However, in the knock-in worms, SWM-1::mCherry was visible in most or all body wall muscles, rather than being restricted to the posterior region. Within cuboidal cells, SWM-1::mCherry was contained within large vesicles closely associated with the apical membrane (Figure 2.2D,E and supplementary data), which are not well-characterized, but have been shown to contain seminal fluid (Smith and Stanfield, 2011) (Lints and Hall, 2009) (Kimble and Hirsh, 1979). In muscle cells, we observed SWM-1::mCherry at low levels that showed no obvious pattern of subcellular localization (Figure 2.2C). Interestingly, in *jn62* males, SWM-1(EEtoVK)::mCherry was visible in muscle at higher levels and at an earlier developmental stage as compared to the wild-type fusion protein (supplementary data),

suggesting that its secretion might be impaired. The SWM-1::mCherry protein also localized to tissues where we did not detect *swm-1* expression. It was present throughout the body cavity (pseudocoelom) and in coelomocytes (Figure 2.2H,I), which are large mesodermal cells that reside within the body cavity and take up soluble material (Fares and Greenwald, 2001; Zhang et al., 2001). Most notably, SWM-1::mCherry was present in the seminal vesicle, as well as the valve region in young adults (Figure 2.2F,G and supplementary data).

SWM-1::mCherry surrounded sperm throughout the lumen of the seminal vesicle. Concentrations were visible surrounding spermatocytes in the final stages of meiosis, which reside in the distal region. Lower levels were present throughout the seminal vesicle surrounding haploid spermatids (Figure 2.2F,G), though this pool of SWM-1::mCherry was often difficult to detect. We surmised that this might be due to lack of space between spermatids, which are tightly packed together. To facilitate visualization of SWM-1::mCherry, we analyzed its localization in a *spe-6(hc163)* mutant, which contains sperm that activate independently of *swm-1* (Muhlrad and Ward, 2002). When activated sperm are present, the contents of the seminal vesicle are more disorganized, with empty spaces between cells. In *spe-6* males, SWM-1::mCherry was present throughout the entire seminal vesicle (supplementary data), indicating that it can spread throughout.

Together with analysis of the transcriptional reporter, these data indicate that SWM-1 is produced in the somatic muscle and vas deferens cuboidal cells. Furthermore, it is secreted from one or both of these tissues and can access the extracellular space around stored sperm.

SWM-1 and TRY-5 localize near sperm independently of each other

In the absence of SWM-1, TRY-5 is visible at elevated levels in the seminal vesicle (Smith and Stanfield, 2011). Since both proteins are secreted into the extracellular space, a simple regulatory mechanism to envision is that SWM-1 inhibits TRY-5 activity *via* a direct interaction. However, alternative models are that SWM-1 might affect the localization, release, or stability of TRY-5.

To address these models, we tested the possibility that TRY-5 and SWM-1 regulate one another's localization. In a *swm-1* mutant background, we observed TRY-5::GFP in the seminal vesicle, as before (Smith and Stanfield, 2011); furthermore, we observed no obvious change in TRY-5::GFP , as might be predicted if SWM-1 were responsible for keeping TRY-5 levels low in this region. Therefore, we tested if the apparent expansion of TRY-5::GFP into the seminal vesicle is due to the loss of *swm-1*, or rather if TRY-5::GFP protein is simply more evident when activated sperm are present. To facilitate visualization of TRY-5 without disrupting endogenous SWM-1, we again utilized a *spe-6* mutant that contains activated sperm. We found that TRY-5::GFP spreads into the seminal vesicle of *spe-6; swm-1(+)* males, just as it does in *spe-6; swm-1(-)* males (Figure 2.3A-F).

We next compared the distributions of SWM-1::mCherry and TRY-5::GFP in wild-type animals. SWM-1::mCherry was expressed broadly, completely surrounding the seminal vesicle in the pseudocoelom and also surrounding sperm in the seminal vesicle. By contrast, TRY-5 is largely restricted to the vas deferens, both in the valve and in cuboidal cells, and is only present at low levels in the posterior of the seminal vesicle (Figure 2.3G,H). Interestingly, we observed SWM-1 and TRY-5 co-localization in

cuboidal cells, suggesting that SWM-1, like TRY-5 is a seminal fluid protein (Figure 2.3 I-L). Lastly, in a *try-5* mutant background, the distribution of SWM-1::mCherry showed no differences as compared to wild-type (Figure 2.3M-P and Figure 2.2B-E).

Thus, SWM-1 per se does not prevent the spread of TRY-5 into the seminal vesicle. Rather, these data suggest a model in which TRY-5 is continually present in the seminal vesicle, though presumably at levels low enough to be inhibited by SWM-1.

Muscle-derived SWM-1 regulates sperm activation within the gonad

To understand how males prevent premature sperm activation, we sought to determine which source(s) of SWM-1 are required for this regulation. We performed tissue-specific expression of SWM-1 and assayed for rescue of the premature activation phenotype of the *swm-1(me87)* null mutant. We generated transgenic strains expressing SWM-1 or SWM-1::mCherry driven by either the vas deferens cuboidal cell-specific *clec-197* promoter ("*Pvas*") or the muscle-specific *myo-3* promoter ("*Pmyo*") (Thoemke et al., 2005) (Fire et al., 1990).

When SWM-1 or SWM-1::mCherry was expressed only in the vas deferens cuboidal cells, it rescued the premature activation of young adult 24 hr post L4 *swm-1(me87)* mutants (Figure 2.4A). However, by 48 hr post L4, sperm were activated in most animals, indicating that this expression was not sufficient to inhibit activation later in adulthood (Figure 2.4A). To determine if this partial rescue activity correlated with differential localization of SWM-1 in the seminal vesicle at early versus later stages, we examined *Pvas*::SWM-1::mCherry localization. Beginning at the L4 stage, SWM-1::mCherry was visible in the lumen between the cuboidal cells and seminal vesicle,

where it contacted spermatids (Figure 2.4B,C and supplementary data). As expected, SWM-1::mCherry localized to cuboidal cell vesicles (Figure 2.4D,E) Interestingly, we also observed a low level of vas deferens-derived SWM-1::mCherry in coelomocytes, despite absence of expression in coelomocytes from this promoter (supplementary data), indicating that SWM-1::mCherry derived from the vas deferens can enter the body cavity. Lastly, we observed SWM-1::mCherry in the seminal vesicle at low levels and at a low frequency (5-20% of animals) (supplementary data). This variability was consistent with the lower rates of rescue we observed for this transgene, and we suspect that the rescue we observed at 24 hr post L4 is due to the vesicles being in contact with sperm early, at the L4 stage (Figure 2.4B,C).

Surprisingly, when expressed in muscle, SWM-1 and SWM-1::mCherry strongly rescued activation in *swm-1(me87)* mutants (Figure 2.4A). *Pmyo*::SWM-1::mCherry was present in body wall muscle, the body cavity, and coelomocytes at all stages analyzed (supplementary data) (Figure 2.4F,G). Similar to its localization in SWM-1::mCherry knock-in animals, SWM-1::mCherry was present around developing and stored sperm in the seminal vesicle (Figure 2.4F,G). Removing the secretion signal resulted in the accumulation of SWM-1::mCherry inside muscle cells and eliminated rescue (Figure 2.4A, H-I). These data suggest that muscle secrete SWM-1 into the body cavity, from where it enters the gonad to inhibit activation.

We reasoned that if the gonad takes up SWM-1 from its surroundings, then secretion into the body cavity from an ectopic source might also be sufficient to inhibit activation. To test this, we used a *dpy-7* promoter to express SWM-1::mCherry from hypodermis, *("Phyp")*, epithelial cells that encase the worm (Gilleard, 1997). We found

that hypodermis-derived SWM-1::mCherry also rescued activation, although at reduced levels (Figure 2.4A). Consistent with the low level of rescue, we observed hypodermis-derived SWM-1::mCherry in the body cavity but not in the seminal vesicle (Figure 2.4J-K, supplementary data).

These experiments strongly support the model that extragonadal, muscle-derived SWM-1 is sufficient to inhibit precocious activation by being secreted into the body cavity where it can enter the seminal vesicle. On the other hand, expression in the gonad is not sufficient, although it appears to contribute to regulation, perhaps via a similar route of delivery.

Proteins move between the pseudocoelom and gonad

We sought to determine whether movement of proteins between the soma and the germline environment was specific to SWM-1, or whether other secreted proteins could enter and exit the gonad. To test this, we expressed secreted mCherry or secreted GFP in specific gonadal and extragonadal tissues, including hypodermis, muscle, neurons, and two regions of the vas deferens: cuboidal cells and valve cells. To assess entry into the gonad, we scored for localization to the seminal vesicle lumen in *spe-6* males, and to assess entry of proteins into the body cavity, we scored for localization in coelomocytes.

We found that mCherry and GFP secreted from extragonadal muscle were present in the pseudocoelom and entered the gonad (Table 2.1). As a functional test, we also expressed TRY-5::GFP outside the gonad and assayed its localization and ability to rescue the *try-5(tm3813)* null mutation. Interestingly, TRY-5::GFP secreted from either muscle or neurons rescued activation in the seminal vesicle (Table 2.1). Furthermore, we

found that the opposite direction of movement of proteins was possible: mCherry secreted from the somatic gonad can be detected in the body cavity. Although we did not observe this gonad-to-pseudocoelom movement for GFP, these data show that the exchange of proteins between the gonad and other tissues is bidirectional and neither specific to SWM-1 nor limited to the sperm activation pathway. In addition, they support our findings that if a protein is present in the body cavity, it can enter the gonad and affect sperm.

SWM-1 is a seminal fluid protein

Since SWM-1 is produced in cuboidal cells of the vas deferens, which release TRY-5 during mating (Figure 2.3I-L), we looked to see if SWM-1 might also be a component of seminal fluid. The cuboidal cells contain a population of variably sized membrane-bound vesicles, which can be observed either using DIC or by visualizing TRY-5::GFP (Figure 2.3I,K). We observed that SWM-1::mCherry and TRY-5::GFP both were present in these vesicles. In a subset, they co-localized, but their relative levels varied (Figure 2.3I-L). To test for transfer, we crossed *[Pswm-1::swm-1::mCherry]* males to wild-type hermaphrodites. During mating, SWM-1::mCherry was secreted and transferred to the hermaphrodite, where it spread throughout the uterus (Figure 2.5A,B). Thus, SWM-1 is contributed to the migratory path of sperm via transfer in seminal fluid.

SWM-1 is produced in muscle and enters the reproductive tract in hermaphrodites

Previous work demonstrated that *swm-1* is not required for hermaphrodite fertility, but is apparently present and functional (Stanfield and Villeneuve, 2006), so we

examined our *swm-1* reporters in adult hermaphrodites. For tissues that are present in both sexes, hermaphrodites appeared similar to males. *Pswm-1::mCherry::H2B* was transcribed in body wall muscle cells (supplementary data), and SWM-1::mCherry was present at low levels in muscle as well as in the body cavity, where it was taken up into coelomocytes (supplementary data). We also observed SWM-1 in the hermaphrodite gonad. *Pswm-1::mCherry::H2B* was expressed in the spermathecae, the regions of the hermaphrodite gonad where sperm are stored (Figure 2.5C-D), and SWM-1::mCherry protein localized throughout the hermaphrodite reproductive tract. Within the lumen of the spermatheca, it surrounded stored sperm (Figure 2.5E,F); it also was present throughout the uterus, among fertilized eggs. As it can be difficult to distinguish between the body cavity and uterus, we used GFP-labeled seminal fluid to mark the lumen of the uterus. *swm-1(jn62[swm-1::mCherry]* hermaphrodites mated to males expressing TRY-5::GFP (Figure 2.5E-H) showed clear co-localization of SWM-1::mCherry and TRY-5::GFP in the uterus. Thus, SWM-1 is present throughout the sperm migratory environment in hermaphrodites. Furthermore, as in males, SWM-1 is secreted into the body cavity and then taken up into the hermaphrodite gonad (data not shown).

Our data suggested that SWM-1 might have a postmating role in the sperm migratory environment. Not only is it contributed to the uterus by both sexes, but its presence in the male gonad is dispensable for regulating activation within the male. Therefore, we sought to determine whether SWM-1 in the hermaphrodite uterus affects male fertility or sperm functions.

Since it is transferred from the male in seminal fluid, we hypothesized that SWM-1 is important for male sperm success. Therefore, we used our strains expressing *swm-1*

in specific tissues to manipulate the level of SWM-1 provided by the hermaphrodite and/or male. To create males that do not transfer SWM-1 (Vas-), we used *[Pmyo-3::swm-1]; swm-1(me87)* males, in which muscle-derived SWM-1 rescues the sperm activation defect (Figure 2.4A,F,G). To create males that transfer an elevated amount of SWM-1, we used *[Pvas-3::swm-1]; swm-1(+)* males (Vas++), in which SWM-1 is expressed from not only the wild-type locus, but also by the Pvas transgene. To eliminate possible complications of hermaphrodite self fertility, we used a *fog-2(q71)* mutation to prevent sperm production in recipient hermaphrodites. We found no difference in male fertility between wild-type males and either Vas++ or Vas- males crossed to *swm-1(-)* hermaphrodites (Figure 2.6A,B). However, Vas++ males had increased fertility in crosses to *swm-1(-)* hermaphrodites as compared to crosses to *swm-1(+)* hermaphrodites (Figure 2.6B). Taken together, these results fail to demonstrate a requirement for SWM-1 in the hermaphrodite reproductive tract, but do suggest that high levels might decrease sperm success. The significance of this result *in vivo* is unclear. Regardless, SWM-1 is clearly a major component of the seminal fluid that males transfer to hermaphrodites during mating.

Discussion

Regulation of sperm development by somatic tissues

Sperm maturation is a critical developmental process regulated by cues derived from multiple somatic cell types. Here we characterize the expression and protein localization of SWM-1, the negative regulator of *C. elegans* sperm activation. We determined that it is produced by somatic body wall muscle and the vas deferens. We

show that the source of SWM-1 in within the gonad, vas deferens cuboidal cells, contributes to regulating motility but is not sufficient to inhibit activation throughout adulthood. Surprisingly, secretion of SWM-1 from extra-gonadal somatic muscle cells is sufficient to inhibit activation. This finding, that a factor critical for fertility is derived from extra-gonadal muscle cells is unexpected. While regulation of sperm development by factors secreted by the vas deferens or other somatic gonad cells is common in other organisms, a role for muscle in sperm development has not, to our knowledge, been identified. However, our data contributes to an emerging body of work showing that muscle is an important source of secreted factors that act on various tissues and that the role of muscle goes beyond structure (Iizuka et al., 2014). Myokines, factors secreted from muscle cells, have been shown to be important in tissues such as vasculature and bone. Additionally, differentiation of *Drosophila* intestinal stem cells is regulated by factors secreted by smooth muscle (Lin et al., 2008).

Using tissue-specific expression, we show that muscle-derived SWM-1 accumulates in pools near sperm that have recently completed meiosis, similar to *swm-1::mCherry* expressed from its native genomic locus. By contrast, cuboidal cell-derived SWM-1::mCherry is not present in these pools. Cuboidal cells are at the proximal end of the gonad, at some distance from newly developed spermatids, and do not complete development until after some sperm has accumulated. Furthermore, it is interesting to note that proteins with a secretion signal expressed in the vas deferens are sequestered in large apical vesicles that are depleted during mating and little appears to be constitutively released, yet the same secreted proteins expressed in muscle are not sequestered but are continually secreted. We speculate that perhaps production of SWM-1 by a cell type that

develops well before sperm is made, that does not sequester secreted proteins or get depleted upon mating, ensures that sufficient inhibitory signal is present immediately after sperm complete meiosis and become competent to activate. This may be important for male fertility in wild populations as it has been shown that males that have mated display increased levels of activation as compared to virgins (Ward et al., 1983). Therefore, muscle as a source of inhibitory signal may help maintain a pool of non-activated sperm and ensure fertility during multiple matings.

Soma-germ line communication

Other proteins secreted from extra-gonadal tissues are critical for fertility. For example, long-distance soma-to-germline transfer has been demonstrated in mice, in which the protease inhibitor Fetuin-B is secreted from the liver but required by oocytes to prevent premature zona pellucida hardening (Dietzel et al., 2013) (Denecke et al., 2003). In *C. elegans*, production and loading of yolk proteins into oocytes occurs *via* uptake from the body cavity, similar to SWM-1. Yolk is produced in the intestine, secreted into the body cavity, and taken up by receptor-mediated endocytosis *via* a yolk protein receptor (Grant and Hirsh, 1999; Kimble and Sharrock, 1983). Our findings differ in that movement of SWM-1::mCherry and other proteins into the gonad does not seem to require a specific transporter. Further experiments to understand how SWM-1 physically enters the seminal vesicle would be interesting for understanding how these factors are exchanged.

Our investigation of SWM-1 demonstrates a curious phenomenon: systemic factors present in the body cavity can enter the gonad and, *vice versa*, factors produced in

the gonad can enter the body cavity. By analyzing mCherry and GFP secreted from within the gonad and from outside the gonad, we demonstrated that this exchange is not specific to sperm activation proteins and that other proteins can also act this way, implying that exchange of factors is widespread. This finding implies that sperm are exposed to a variety of factors in the pseudocoelom suggesting that sperm cells are robust in their ability to encounter many signaling environments.

Regulation of sperm development by protease signaling

Our analysis of the localization of SWM-1 and TRY-5 in combination is a step toward understanding the molecular mechanism by which they regulate sperm activation. By using *spe-6* mutants, we showed that TRY-5 is able to spread into the seminal vesicle even in the presence of SWM-1. We also showed that the opposite is true: localization of SWM-1 does not depend on TRY-5. Furthermore, visualizing SWM-1::mCherry in combination with TRY-5::GFP suggests that males regulate activation mainly by expressing the activator and inhibitor in different domains; whereas TRY-5 is mainly sequestered in the vas deferens, SWM-1 is widespread, surrounding the entire seminal vesicle in the pseudocoelom where it can freely enter the gonad and keep activation in check. It is interesting that SWM-1 and TRY-5 are in close proximity in the seminal vesicle. This suggests that where TRY-5 does encounter SWM-1, they might directly interact.

These data show that SWM-1 does not inhibit TRY-5 release from the valve. Instead, they support a model where TRY-5 is secreted into the seminal vesicle at a low basal rate that is not sufficient to activate sperm, due to the predominantly inhibitory

environment maintained by consistent secretion of SWM-1 from somatic muscle cells that surround the seminal vesicle. Although these findings do not define the molecular mechanisms of SWM-1 and TRY-5, they are consistent with a direct interaction in the extracellular environment. Indeed, *in vitro* biochemical analysis of recombinant SWM-1 and TRY-5 activities would be interesting future work. Nevertheless, these co-localization studies highlight the utility of *C. elegans* as a model for studying the sperm signaling environment and measuring the production and release of seminal fluid proteins in the context of live animals.

SWM-1 in the sperm migratory environment

In the hermaphrodite, SWM-1 is present, yet its role has been elusive. While it is known that *swm-1* is functional in the hermaphrodite, it is dispensable for fertility (Stanfield and Villeneuve, 2006). Our analysis of SWM-1::mCherry in males and hermaphrodites suggests that it is important in the sperm migratory environment as it is contributed both by males in seminal fluid and is also supplied by hermaphrodites to the uterus. Using males that lack SWM-1 in the vas deferens but have SWM-1 in muscle to inhibit premature activation, we showed that male fertility is not affected by SWM-1 in seminal fluid but is affected by hermaphrodite SWM-1. Although our experiments failed to identify a definitive role for SWM-1 in the hermaphrodite reproductive tract, it is possible that our assay was not sufficiently sensitive to detect an effect of SWM-1. Given that SWM-1 plays a critical role for male but not hermaphrodite fertility, perhaps its effect in the uterus on fertility is subtle and its role may only be uncovered in more stringent conditions such as when sperm from multiple males compete.

In summary, these studies have uncovered a critical yet surprising role for muscle in the regulation of *C. elegans* sperm development. Additionally, this work demonstrates that there is significant communication between extragonadal tissues and the gonad in both males and hermaphrodites, which has implications for understanding not only sperm activation but virtually every reproductive process in the adult.

Materials and methods

C. elegans genetics

C. elegans were grown at 20ºC on nematode growth medium seeded with *Escherichia coli* strain OP50 and were derived from the wild-type Bristol N2 strain (Brenner, 1974). To obtain males, the *him-5(e1490)* allele was present in all strains where males were analyzed, including wild-type controls (Hodgkin et al., 1979). Recombination was used to introduce the *him-5(e1490)* allele into the *swm-1(jn60[swm-1(EEtoVK)::mCherry])* strain. Other alleles used for experiments were *ttTi5605, dpy-18(e364), spe-6(hc163), unc-119(ed3), cxTi10816, dpy-11(e224), try-5(tm3813), swm-1(me87), fog-2(q71),* and *ajm-1::GFP* (r-Jensen et al., 2008) (Frøkjær-Jensen et al., 2012) (Lee et al., 2017).

Transgenic strains

Mos-mediated single-copy insertion (MosSCI) was used to integrate transgenes at the *ttTi5605 II* and *cxTi10816 IV* loci as described by Frokjaer-Jensen (r-Jensen et al., 2008) (Frøkjær-Jensen et al., 2012). Two or more independent lines were analyzed for each construct.

MosSCI donor constructs were generated using the MultiSite Gateway Three-fragment Vector Construction Kit (Thermo Fisher Scientific). Fragments were recombined into the pCFJ150 or pCFJ212 destination vectors for transgene insertion on chromosome II or IV, respectively (r-Jensen et al., 2008). A description of fragments used for generating targeting constructs is listed in the supplementary data and primers used to generate donor vector fragments are listed in the supplementary data.

To select an appropriate *swm-1* promoter, we generated transgenes that included different lengths of sequence 5' and 3' to the *swm-1* coding region and assayed their ability to rescue *swm-1(me87)*, a null allele. Transgenes containing 1.3 kb of 5' sequence and 0.7 kb of downstream sequence showed strong levels of rescue of the premature activation phenotype (supplementary data). However, 0.7 kb of 5' sequence and 0.2 kb of 3' sequence regions showed greatly reduced rescue (data not shown).

Molecular biology

MosSCI donor constructs were generated using the MultiSite Gateway Three-fragment Vector Construction Kit (Thermo Fisher Scientific) to recombine three vector fragments into the pCFJ150 or pCFJ212 for transgene insertion on chromosome II or IV respectively. pCFJ150, pCFJ212 and the body wall muscle promoter vectors P2305 and pPM4 containing the *myo-3* promoter described by Fire et al. 1990 were gifts from E. Jorgensen. The PADA96 vector containing the *dpy-7* promoter was a gift from L. Johnstone. The mCherry and GFP tagged H2B vectors pCM1.51, pCM1.35 and pCM5.37 were gifts from G. Seydoux (Merritt et al., 2008). A description of fragments used for generating targeting constructs is listed in the supplementary data and primers

used to generate donor vector fragments are listed in supplementary data.

CRISPR/Cas9 genome editing

The co-CRISPR technique described by Kim et al. (2014) and Arribere et al., (2014) was adapted to insert mCherry into the endogenous *swm-1* locus. N2 hermaphrodites were injected with DNA fragments for expression of *swm-1* guide RNA5 and *C25E10.8* guide RNA3 along with thepJA42 and AF-JA-53 reagents for *rol-6* co-CRISPR selection (Arribere et al., 2014)., Simultaneously, we provided a linear DNA repair template to introduce mCherry coding sequences immediately before the *swm-1* stop codon, two silent PAM site mutations to prevent Cas9 re-cutting and 114-124 bp homology arms. Guide RNA DNA fragments were generated by fusion PCR as described by Ward 2015 and pJW1259 (Cas9 expression vector; Ward, 2015). The repair template was amplified with Phusion (New England Biolabs) and purified with the Thermo Fisher PureLink PCR purification kit. PCR assays were used to confirm integration of mCherry, followed by sequencing 3390 bp upstream of the *swm-1* guide RNA5 cut site and 263 bp downstream of the *C25E10.8* guide RNA cut site. The *him-5(jn64)* allele was generated using *him-5* gRNA1 and *him-5* gRNA3 to induce deletions at the *him-5* locus in the *swm-1(jn62[swm-1::mCherry])*.

Quantification of sperm activation

Sperm activation was quantified by collecting virgin males as L4 larvae, incubating them at 20°C for 24 hr or 48 hr, and examining the seminal vesicle region using differential interference contrast (DIC) microscopy. Each animal was scored as

"non-Activated," if it contained only spermatids without visible pseudopods; "sperm + spermatids," if we observed sperm with and without pseudopods; and "Activated," if all sperm within the seminal vesicle had developed pseudopods. For DIC, we used an AxioImager M1 microscope equipped with an AxioCam MRm (Zeiss). Confocal images were acquired using an Olympus FV1000 microscope.

Male fertility assays

Males and hermaphrodites were collected as L4 larvae, incubated at 20°C for 24 hr, and placed together in a 1:1 ratio for 24 hours to allow for mating. Males were then removed and hermaphrodites were transferred to fresh plates every 24 hr for 4 additional days. Matings that produced fewer than 150 total offspring or where offspring were not produced for at least three transfers were excluded from analysis. *dpy-11(e224) swm-1(me87) fog-2(q71)* animals are both male- and hermaphrodite-sterile, so *dpy-11(e224) swm-1(me87) fog-2(q71)/dpy-11(e224) swm-1(jn62[swm-1::mCherry]) fog-2(q71)* heterozygotes were maintained and hermaphrodites lacking mCherry were selected for assays. The *dpy-11(e224) fog-2(q71)* homozygous strain was maintained by crossing males to hermaphrodites.

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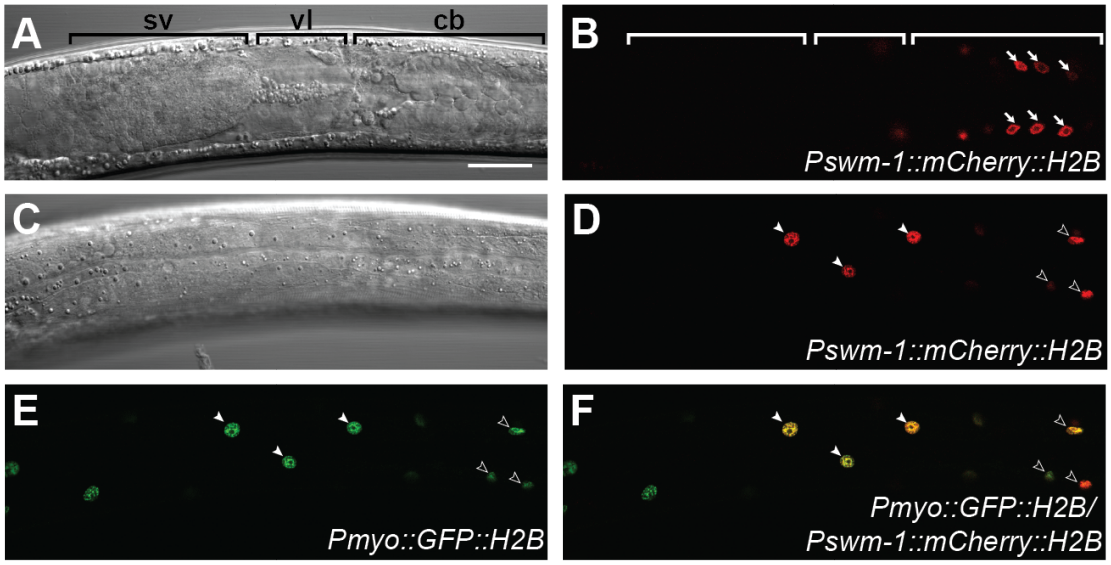

Figure 2.1 *swm-1* is expressed in the somatic gonad and posterior muscle cells. Transmitted-light and confocal images of *jnSi130[Pswm-1::mCherry::H2B]* 24 hr adult males. Ventral views are shown. (A,B) *swm-1* is expressed in vas deferens cuboidal cells. Arrows indicate cuboidal cell nuclei. (C,D) swm-1 is expressed in body wall muscle (arrowheads) and male-specific diagonal muscle (open arrowheads). (E,F) Co-localization with the muscle-specific transcriptional reporter *jnSi226[Pmyo::GFP::H2B]*. Labels: sv, seminal vesicle; vl, valve; cb, vas deferens cuboidal cells. Scale bar: 25 μm. n=30.

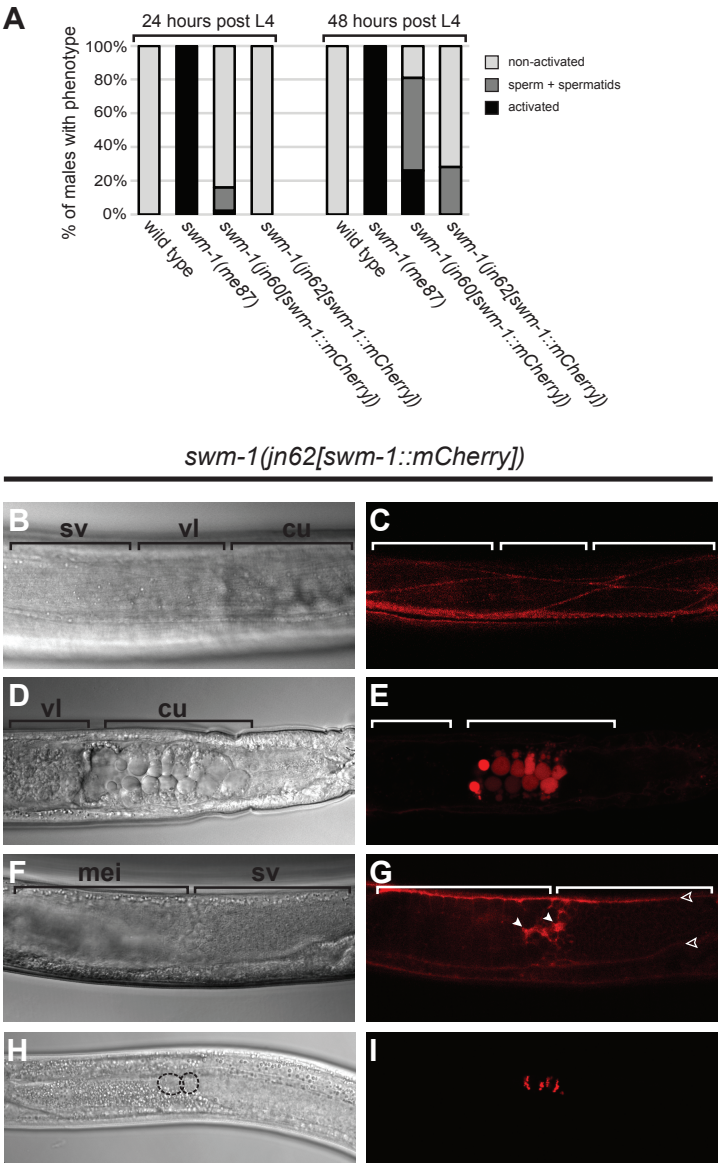

Figure 2.2 SWM-1::mCherry localizes to the somatic gonad, muscle, and seminal vesicle. (A) Quantification of sperm activation of *swm-1(jn62[swm-1::mCherry])* and *swm-1(jn60[swm-1(EEtoVK)::mCherry])* 24 hr post L4 adult males. Males contain either nonactivated (light grey) a mix of sperm and spermatids (grey) or activated sperm (black). Premature activation is increased in *swm-1(jn60)* males as compared to *swm-1(jn62)* n = 24-50. (B-I) Transmitted-light and confocal images of *swm-1(jn62)* 24 hr post L4 males. (B,C) SWM-1::mCherry localizes to muscle cells overlying the male gonad. (D,E) SWM-1::mCherry localizes to intracellular vesicles within a subset of vas deferens cuboidal cells. (F,G) SWM-1::mCherry surrounds the seminal vesicle in the pseudocoelom (open arrowheads) and is concentrated in areas near newly developed spermatids (arrowheads). (H,I) SWM-1::mCherry accumulates in coelomocytes. Labels: mei, meiotic region; sv, seminal vesicle; vl, vas deferens valve cells; and cu, vas deferens cuboidal cells. Scale bar, 25 µm.

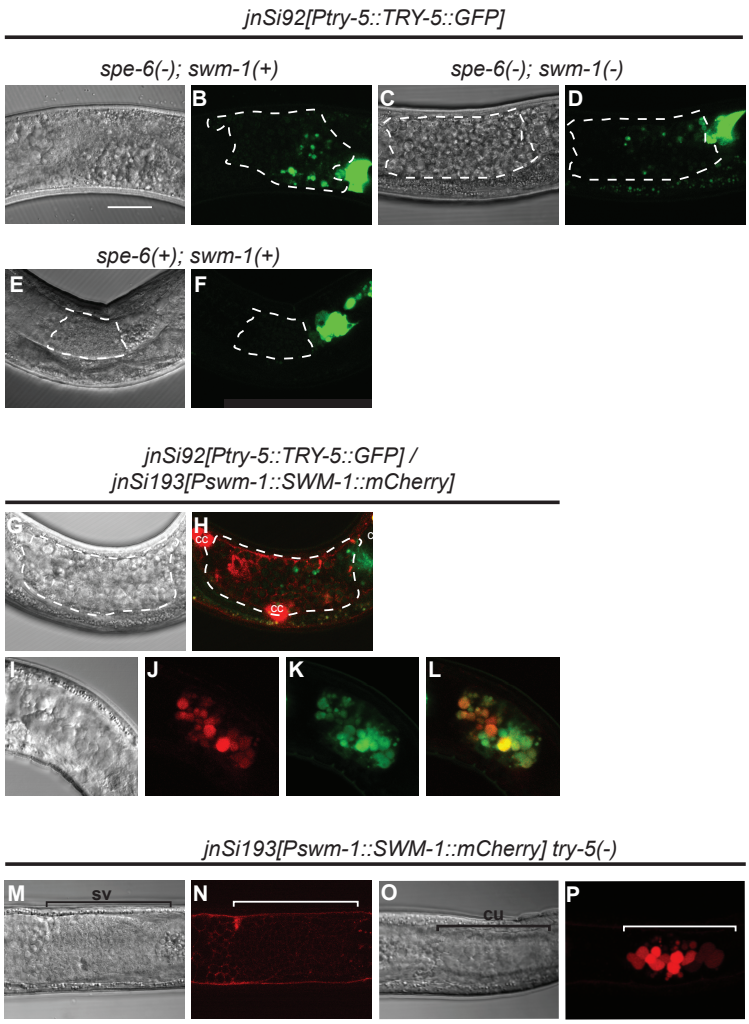

Figure 2.3 SWM-1 and TRY-5 do not regulate localization of each other. (A-H) 24 hr post L4 adult males carrying the *jnSi92[TRY-5::GFP]* transgene in *spe-6(hc163)* and *swm-1(me87)* mutant backgrounds. Outlined regions indicate the seminal vesicle. n = 20-25. Scale bar, 25 µm. (A,B) *spe-6; jnSi92[TRY-5::GFP]; swm-1(+)* male seminal vesicle. TRY-5::GFP spreads into the seminal vesicle independently of *swm-1*. TRY-5::GFP surrounds sperm in the anterior region of the seminal vesicle. (C,D) *spe-6; jnSi92[TRY-5::GFP]; swm-1(-)* male seminal vesicle. TRY-5::GFP similarly spreads into the seminal vesicle surrounding sperm in the anterior region. (E-H) Control *spe-6(+)* animals show normal TRY-5::GFP localization. (E,F) *swm-1(+)* control male. (G,H) *swm-1(me87)* male. (I-N) animals heterozygous for the MosSCI transgenes of SWM-1::mCherry and TRY-5::GFP. (I,J) pseudocoelomic SWM-1::mCherry surrounds the seminal vesicle and is present throughout the seminal vesicle while TRY-5::GFP is sequestered in the valve region. (K-N) SWM-1::mCherry and TRY-5::GFP co-localize in vas deferens cuboidal cell vesicles. (O-R) *jnSi193[Pswm-1::swm-1::mCherry]; swm-1(me87) try-5(tm3813)* males. Localization of SWM-1::mCherry is normal in *try-5(tm3813)* null mutants in the cuboidal cells and seminal vesicle as compared to SWM-1::mCherry (Figure 2.2).

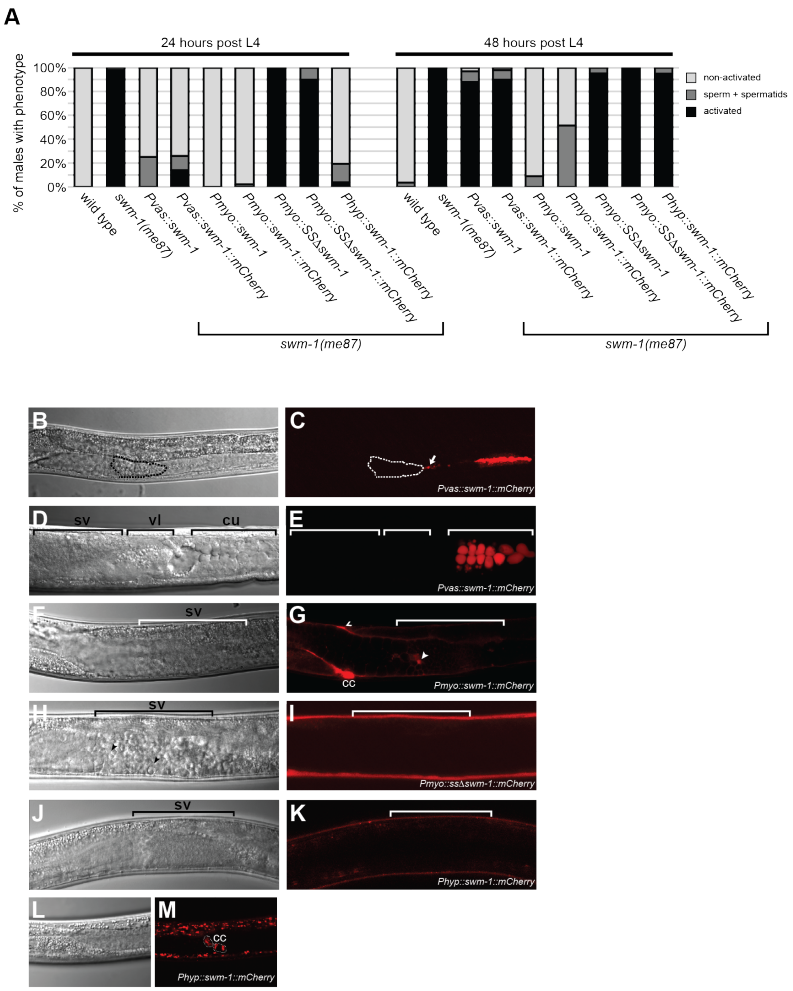

Figure 2.4 Secretion of muscle-derived SWM-1 is sufficient to rescue activation. (A) Quantification of the sperm activation of 24 and 48 hr post L4 *swm-1(me87)* males carrying cuboidal cell, muscle or hypodermis-specific SWM-1, or SWM-1::mCherry transgenes. Males contain either, nonactivated (light grey), a mix of sperm and spermatids (grey), or nonactivated (black) sperm n = 20-47. (B-E) *[Pclec-197::swm-1::mCherry]; swm-1(me87)* males. (B,C) SWM-1::mCherry is near sperm in L4 males. Outlined region indicates the seminal vesicle. Arrow indicates SWM-1::mCherry in close proximity to sperm. (D,E) Cuboidal cell-specific SWM-1::mCherry in 24 hr post L4 males. (F,G) *[Pmyo::swm-1::mCherry]; swm-1(me87)* 24 hr post L4 adult male. Muscle cell-specific SWM-1::mCherry is present in the pseudocoelom and in the seminal vesicle surrounding sperm. Arrow indicates concentrations of SWM-1::mCherry surrounding sperm that have recently completed meiotic division. Open arrowhead indicates SWM-1::mCherry in the pseudocoelom. Coelomocyte, cc. (H,I) *[Pmyo::SSΔ::swm-1::mCherry]; swm-1(me87)* adult male. SWM-1::mCherry is sequestered in muscle and sperm are activated. Arrowheads indicate activated sperm cells. (J-M) *[Phyp::swm-1::mCherry]; swm-1(me87)* adult male. (J-K) Hypodermis-derived SWM-1::mCherry is not detectable in the seminal vesicle. (L,M) Hypodermis-derived SWM-1::mCherry is secreted into the body cavity and is present in coelomocytes.

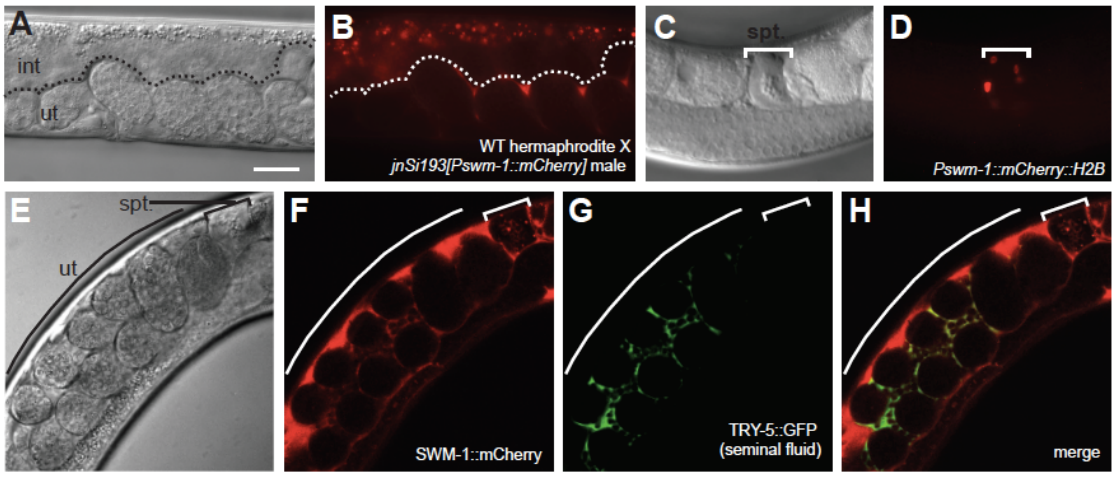

Figure 2.5 SWM-1 is present in the hermaphrodite uterus. (A,B) Uterus of a 24 hr post L4 adult hermaphrodite that has mated with a *jnSi193[Pswm-1::swm-1::mCherry]; swm-1(me87)* male. Males contribute SWM-1 to the hermaphrodite uterus in seminal fluid. Male-derived SWM-1::mcherry is present between fertilized eggs in the hermaphrodite uterus. (C,D) *jnSi130[Pswm-1::mCherry::H2B]* 24 hr post L4 hermaphrodite. *swm-1* is expressed in spermathecal cells. (E-H) Uterus of 24 hr post L4 *swm-1(jn62[swm-1::mCherry])* hermaphrodite after mating with a *jnSi92[Ptry-5::try-5::gfp]; swm-1(me87) try-5(tm3813) male*. SWM-1::mCherry co-localizes with male seminal fluid in the hermaphrodite uterus. SWM-1::mCherry is present in the spermatheca surrounding stored sperm. Regions of the hermaphrodite reproductive tract labeled: int, intestine; ut, uterus; spt, spermatheca.

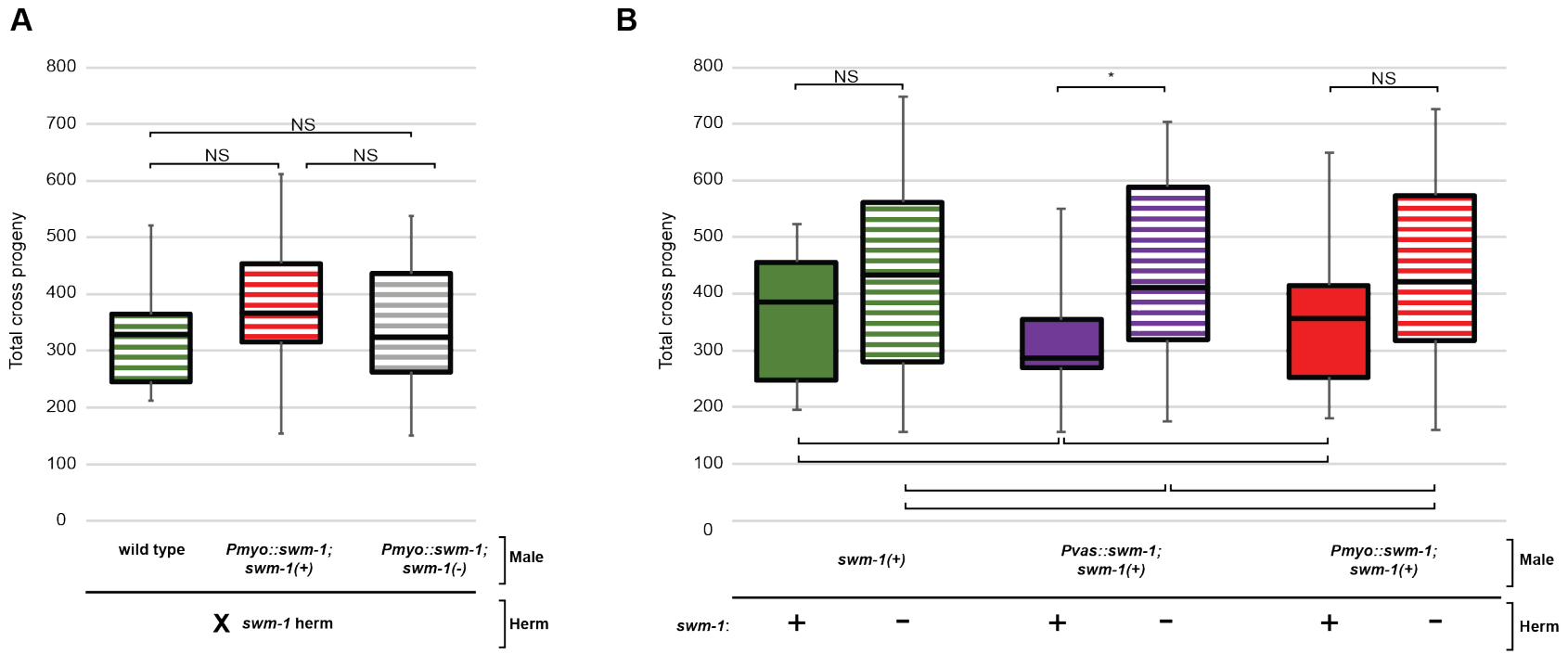

Figure 2.6 The level of SWM-1 in the hermaphrodite affects male fertility. (A) Male fertility is unaffected in males that lack vas deferens SWM-1. Control *[Pmyo::swm-1]; swm-1(+)* and *[Pmyo::swm-1]; swm-1(-)* males were mated to *fog-2 swm-1(-)* hermaphrodites and total cross progeny is shown. (B) Overexpression of SWM-1 reduces male fertility and fertility is restored by eliminating SWM-1 in the hermaphrodite. Males overexpressing SWM-1 in either the vas deferens (purple) or muscle(red) were crossed to *swm-1(+)* or *swm-1(-) fog-2* hermaphrodites and total cross progeny is shown. Thick bar represents median. *, $p < 0.05$; NS, not significant (Kolmogorov-Smirnov test).

Table 2.1 Proteins can move into and out of the gonad. Protein localization of secreted mCherry, GFP, SWM-1::mCherry and TRY-5::GFP expressed under different promoters in extragonadal tissues (muscle, hypodermis, or neurons) or gonadal vas deferens cells (valve or vas deferens). Presence (+) or absence (-) of localization to the indicated tissue is shown.

	Tissue of origin	Secreted protein	Coelomocytes	Seminal vesicle	Rescues activation?
Extra-gonadal	muscle	mCherry	+	+	NA
	muscle	GFP	+	+	NA
	muscle	SWM-1::mCherry	+	+	+
	hypodermis	SWM-1::mCherry	+	-	+
	muscle	TRY-5::GFP	+	+	+
	neurons	TRY-5::GFP	-	-	+
Gonadal	vas deferens cuboidal cells	mCherry	+	-	NA
	vas deferens cuboidal cells	GFP	-	-	NA
	vas deferens valve cells	mCherry	+	-	NA
	vas deferens valve cells	GFP	-	-	NA
	vas deferens cuboidal cells	SWM-1::mCherry	+	-	partial
	vas deferens cuboidal cells	TRY-5::GFP	-	-	low

Supplementary Data

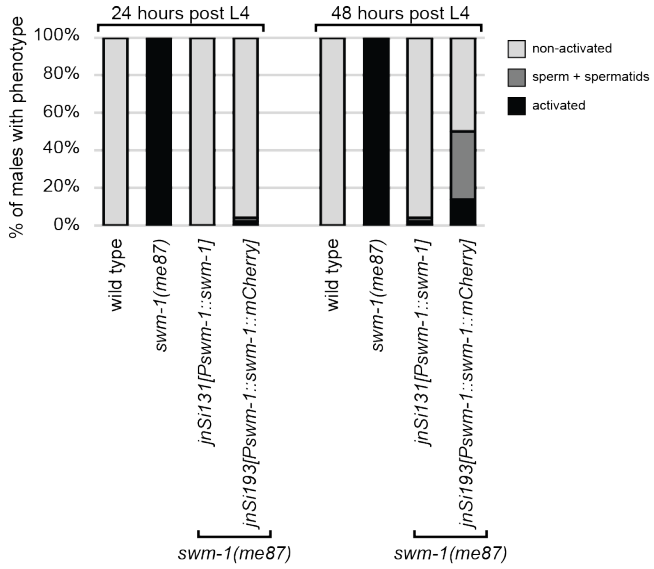

Figure 2.S1 Single-copy insertion swm-1 transgenes rescue the premature sperm activation of swm-1(me87) null mutants. Quantification of sperm activation phenotype of transgenic males carrying either *swm-1* or *swm-1::mCherry* single-copy insertion transgenes. Columns show the percentage of males with the indicated phenotypes. n = 29-57.

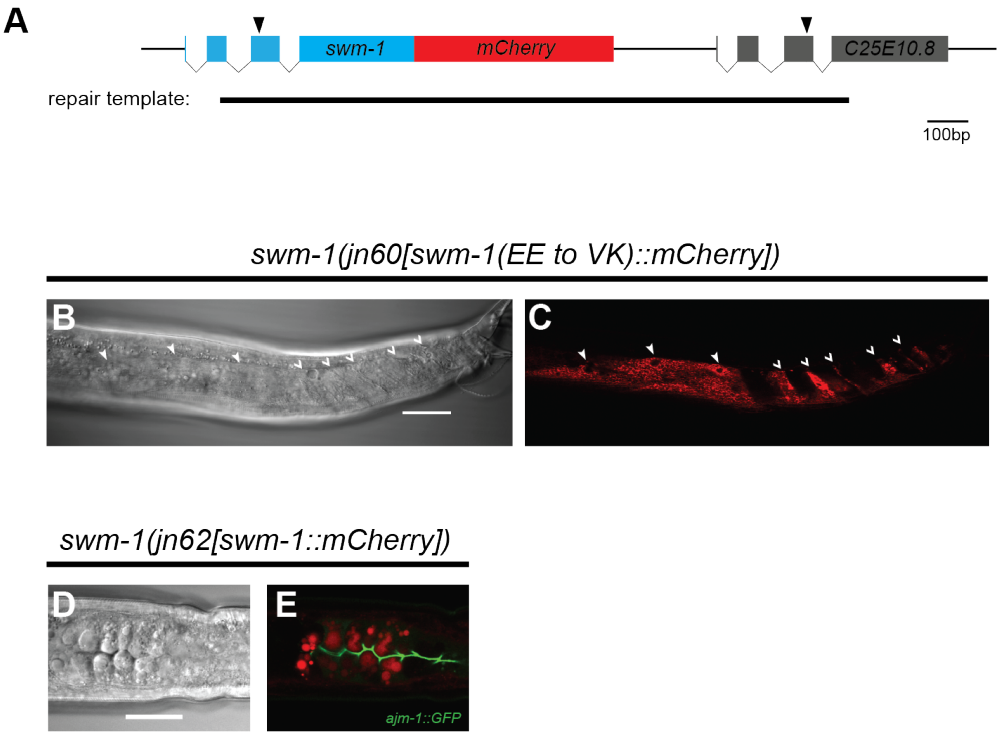

Figure 2.S2 CRISPR design and subcellular SWM-1 localization. (A) Schematic of CRISPR strategy to generate genomic *swm-1::mCherry*. Arrowheads indicate Cas9 cut sites. The repair template included 114 bp of sequence 5' to the *swm-1* gRNA cut site, mCherry fused to the C-terminus of *swm-1,* 124 bp of sequence 3' to the *C25E10.8* gRNA cut site, and two silent PAM site mutations. (B,C) Images of a 24 hr post L4 *swm-1(jn60[swm-1(EEtoVK)::mCherry])* adult male. SWM-1 accumulated in somatic body wall muscle cells (arrowheads) and male-specific diagonal tail muscle cells (open arrowheads). (D,E) Images of a 24 hr post L4 *swm-1(jn62[swm-1::mCherry])* adult male with the apical membrane marker AJM-1::GFP. SWM-1::mCherry-containing vesicles are closely associated with the apical membrane.

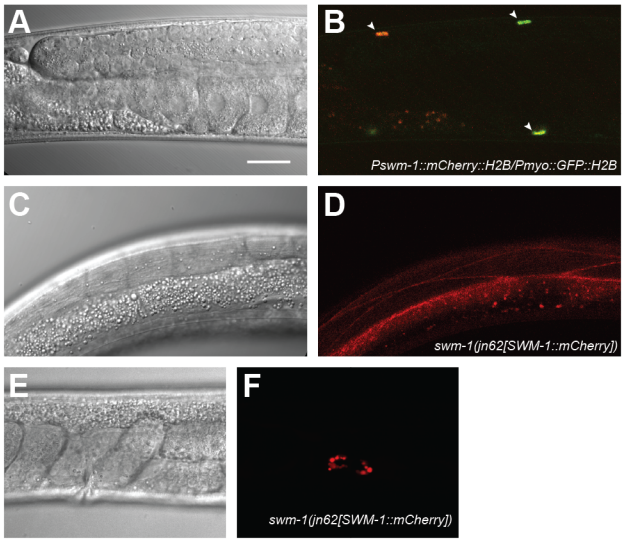

Figure 2.S3 SWM-1 is present in hermaphrodite muscle and pseudocoelom. A) Co-localization of *[Pswm-1::mCherry::H2B]* with *[Pmyo::GFP::H2B]*. *swm-1* is expressed in body wall muscle. B-F) *swm-1(jn62[SWM-1::mCherry]* 24 hour hermaphrodite SWM-1::mCherry localization in muscle and coelomocytes.

Table 2.S1 Sperm activation phenotype and localization of SWM-1::mCherry transgens. Localization of SWM-1 at larval and adult stages. Localization is indicated by presence, +, absence – or as the % of animals with expression in the indicated tissue. 0 indicates animals of that stage were not analyzed. Letters in extra gonadal category indicate muscle, M or hypodermis H.

transgene	phenotype: % Activated - Sperm + Sptds. - Nonactivated		cuboidal cells			proximal seminal vesicle			distal seminal vesicle			pseudocoelom			coelomocytes						extra gonadal					
	24	48	L4	24 hr	48hr	L4	24 hr	48hr	L4	24 hr	48hr	L4	24 hr	48hr	L1	L2	L3	L4	24 hr	48hr	L1	L2	L3	L4	24 hr	48hr
transcriptional reporter	0-0-100	0-0-100	+	+	+	-	-	-	-	-	-	NA	NA	NA	-	-	-	-	-	-	-	-	1-2 cells 46% M	1-6 cells M	7-11 cells M	9-13 cells M
[SWM-1::mCherry] (mosSCI)	2-2-96	14-34-52	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-
[SWM-1::mCherry]	0-0-100	0-27-72	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+ M	+ M	+ M
[SWM-1::mCherry] *spe-6(-)*	100-0-0	100-0-0	0	+	+	0	+	+	0	+	+	0	+	+	0	+	+	0	0	0	0	+	+ M	0	0	0
SWM-1 (EE to VK)::mCherry	2-14-84	26-55-19	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+ M	+ M	+ M	+ M
[pVas::SWM-1::mCherry]	14-12-74	88-8-2	+	+	+	-	19%	5%	-	-	-	-	+	+	-	-	35%	+	+	-	-	-	-	-	-	-
[pMyo::SWM-1::mCherry]	0-4-96	0-50-50	NA	NA	NA	-	9%	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+ M	+ M
[pMyo::SSΔ::SWM-1::mCherry]	89-10-0	100-0-0	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	-	-	-	0	0	0	-	+ M	+ M
[Phyp::swm-1::mCherry]	89-15-3	95-5-0	0	-	-	0	-	-	0	-	-		+	+	0	0	0	0	+	+	0	0	0	0	+ H	+ H

Table 2.S2 List of oligonucleotides for generating entry vectors used in targeting constructs

Fragment description	Fragment length	Forward primer	Reverse primer	Entry Vector	Plasmid name
swm-1 promoter	1365 bp	5'-GGGGACAACTTTGTATAGAAAAG TTGTGCCCATTTCTCACACAGAC	5'-GGGGACTGCTTTTTTGTACAAACTTG TTTCACAATAACGAAGGGCAAC	pDONR P4-P1R	pAKS1
swm-1 coding region	630 bp	5'-GGGGACAAGTTTGGTACAAAAAAG CAGGCTTGAAAATGGTGAGTTTTTGAG	5'-GGGGACCACTTTGTACAAGAAAGCTGG GTATTATTTTGGACAATCTTTCTTATCAATG	pDONR 221	pAKS2
swm-1 3' region	729 bp	5'-GGGGACAGCTTTCTTGTACAAAGTG GTTTGTTTTTGAGTAAACATTTCCAAG	5'-GGGGACAACTATGTATAATAAAG TTGACGCACAAATCCTTTCTTGC	pDONR P2R-P3	pAKS3.

Table 2.S3 List of oligonucleotides used to generate gRNA DNA fragments by fusion PCR

Fragment description	Fragment length	Forward primer	Reverse primer
swm-1 gRNA5	461	5’-CCTCCTATTGCGAGATGTCTTGCA CGATACCAGCTCCTCATGTTTAAGAGCT ATGCTGGA	5’-AAAAATAGGCGTATCACGAGG
C25E10.8 gRNA3	483	5’-CCTCCTATTGCGAGATGTCTTGAT CACTGTGAGACACAATGGTTTAAGAGC TATGCTGGA	5’-AAAAATAGGCGTATCACGAGG
SWM-1::mCherry repair template	2044	5'-TAATCACATGCATCGTTG	5'-ACGCACAAATCCTTTCTTGC

Table 2.S4 List of DNA sequences targeted for CRISPR editing

gene	guide RNA	Target sequence (PAM)
C25E10.8	gRNA3	GATCACTGTGAGACACAATG(CGG)
swm-1	gRNA5	GCACGATACCAGCTCCTCAT(TGG)
him-5	gRNA1	GATGAACAACGAAAAACAGT(TGG)
him-5	gRNA3	AAGTGGATCAGGAATCAGCG(TGG)

CHAPTER 3

GENETIC ANALYSIS OF *SWM-1* TRYPSIN INHIBITOR-LIKE DOMAINS

Introduction

Serine protease inhibitors are important in a wide variety of cellular processes. For example in humans, serine protease inhibitors are critical for regulating proteolysis involved in blood coagulation, inflammation, sperm maturation, and cell migration (Huntington, 2011) (Poiani, 2006) (Torriglia et al., 2017). One class of serine protease inhibitor that has not been extensively studied are the low molecular weight trypsin inhibitor-like (TIL) domain proteins. TIL domains are characterized by 10 conserved cysteine residues that form 5 disulfide bridges important for folding and a 3-4 amino acid reactive site where the inhibitor is cleaved by serine proteases (Babin et al., 1984; Chen et al., 2013; Di Cera, 2009; Peanasky et al., 1984a; Peanasky et al., 1984b). These protease inhibitors have been found in frog skin secretions, *Drosophila* seminal fluid, honeybee hemolymph, and scorpion venom (Lung et al., 2002; Peanasky et al., 1984b; Rosengren et al., 2001; Zeng et al., 2013). They are also present in larger proteins in humans, including otogelin-like and von Willebrand factor (vWF) (Bonnet et al., 2013; Zhou et al., 2012). Importantly, mutations in the TIL domains of vWF cause the blood clotting disorder Von Willebrand disease (Shiltagh et al., 2014). Given their role in a large variety of cellular processes, the study of TIL domains has broad implications in of reproductive biology, blood coagulation and parasitology.

SWM-1 may inhibit two proteases

In *C. elegans,* the negative regulator of sperm activation, SWM-1, contains two TIL domains. The *swm-1(me87)* null mutation causes premature sperm activation, while two other mutants, *swm-1(me86)* and *swm-1(me66),* are partial loss of function alleles isolated in a noncomplementation screen looking for additional *swm-1* alleles (Stanfield and Villeneuve, 2006). The *me66* allele affects the N-terminal TIL domain (N-TIL) while the *me86* allele affects the C-terminal TIL domain (C-TIL), and both are missense mutations. Interestingly, trans-heterozygous *me66/me86* animals show reduced levels of premature activation as compared to either homozygote (Stanfield and Villeneuve, 2006). This indicates that the two alleles partially complement each other. Furthermore, the reactive sites of the TIL domains are divergent (Stanfield and Villeneuve, 2006), suggesting that SWM-1 may inhibit two distinct proteases.

While analysis of the *me86* and *me66* mutants suggests SWM-1 may have two targets, the two mutations are not equivalent and likely have different effects on TIL domain structure and/or catalytic activity. In the N-TIL, the *me86* mutation affects an amino acid adjacent to the 7th conserved cysteine (C7) important for structure, while in the C-TIL, *me66* affects the first conserved cysteine (C1). Therefore, understanding the function of each domain requires further investigation. A more detailed analysis of individual SWM-1 TIL domains will aid in understanding the molecular mechanism by which SWM-1 acts and determine whether SWM-1 may have multiple targets. In this chapter, I describe my investigation of the role of individual TIL domains in inhibiting sperm activation using transgenic animals that contain only on of the two TIL domains.

Results

To determine whether one or both TIL domains were required to inhibit activation, I analyzed single TIL domain transgenic animals using the MosSCI technique to generate single copy-insertion transgenes at the *ttTi5605* locus (r-Jensen et al., 2008). As a control, I also analyzed animals with a full-length *swm-1* transgene that included both TIL domains. As a readout of TIL function, I analyzed the seminal vesicles of adult transgenic males in the *swm-1(me87)* null background to determine whether they were filled with nonactivated sperm, a mixture of sperm and spermatids, or only activated sperm. I looked at two time points when males are healthy adults and have accumulated a significant amount of sperm. I found that the single N-TIL transgene failed to rescue activation at 24 and 48 hours post L4. However, the single C-TIL transgene partially rescued the premature sperm activation of *swm-1(me87)* null mutants in 24 hour but not 48 hour post L4 adults (Figure 3.1). Importantly, the full-length control exhibits full rescue of the null at both 24 and 48 hours. This suggests that neither TIL domain alone is sufficient to inhibit activation, but the C-terminal TIL domain plays a more significant role in inhibiting activation.

To further test the requirement for the TIL domains, I also analyzed their ability to rescue the weaker alleles of *swm-1, me66,* and *me86.* In the *swm-1(me86)* mutant background, both N-TIL and C-TIL transgenes showed strong rescue at 24 hours and partial rescue at 48 hours post L4. In the *swm-1(me66)* mutant background, both transgenes showed strong rescue at 24 hours post L4 and no rescue at 48 hours post L4. Together these results suggest that the C-terminal TIL domain might play a more significant role in inhibiting activation, but both domains exhibit inhibitory activity.

Additionally, the analysis of the weak alleles suggests that the domains can compensate for each other.

Discussion

This analysis of SWM-1 TIL domains suggest that they are not equivalent in their role of inhibiting sperm activation. While these results further support the idea that SWM-1 may have multiple targets, they leave ambiguity as to how SWM-1 functions at the molecular level. Whereas introduction of the C-TIL partially inhibits activation of *swm-1* null mutants, introduction of the N-TIL does not, suggesting that the C-TIL may play a more significant role in activation, although is not sufficient for full regulation. Furthermore, in the *swm-1(me86)* and *swm-1(me66)* genetic backgrounds, the presence of a C-TIL mutation appears to be more damaging than the N-TIL mutation, regardless of which transgene was introduced. The fact that either transgene exhibited similar levels of rescue of the weak alleles also suggests that the domains can partially compensate for each other.

Understanding the molecular mechanism by which SWM-1 inhibits activation would be aided by targeted mutations that affect each TIL domain equally, identification of downstream SWM-1 targets, and/or *in vitro* biochemical assays. Targeted mutations should carefully consider previous biochemical analysis of TIL domains. Specifically, in a study by Chen et al. (2013), chimeras were made of SjAPI, a TIL domain protein that is present in scorpion venom. In this study, researchers used SjAPI as a scaffold and created recombinant proteins, replacing reactive residues with those of other scorpion venom TIL proteins with a known substrate. They found that replacement of the reactive

site alone is not sufficient to target a substrate in the SjAPI chimera (Chen et al., 2013). This indicates that substrate specificity of TIL domains is determined not only by the reactive site but also by other, unknown characteristics of the protein. Given these findings, interpretation of targeted mutation analysis may be challenging and focusing on generating mutations in the conserved cysteines important for folding rather than ablating substrate binding in the reactive site may be more likely to shed light on the molecular mechanism.

Perhaps more fruitful approaches would be to find additional genes in the sperm activation pathway and to test SWM-1 for protease inhibitory activity *in vitro*. While it is known that TRY-5 and the SLC6 family protein SNF-10 are downstream of SWM-1, it remains unknown whether any of these proteins directly interact with each other (Fenker et al., 2014; Smith and Stanfield, 2011). Furthermore, a SWM-1 suppressor screen resulted in the identification of at least 4 genes that suppress premature activation of *swm-1(me86)* mutants, but do not complement *try-5* or *snf-10*. Preliminary analysis of *swm-1* suppressor strains can be found in chapter 4 of this dissertation.

Why would sperm activation be dependent on two TIL domains? Of the known TIL domain-containing proteins, many contain only a single TIL domain. Perhaps in the case of sperm activation, the presence of two TIL domains demonstrates the importance of the process of activation. Indeed, SWM-1 is well conserved among closely and distantly related species of *Caenorhabditis* that utilize both androdioecious (male/hermaphrodite) and gonochoristic (male/female) systems of sex determination (Wei et al., 2014). Furthermore, duplication of TIL domains is common and likely represents a means of genetic innovation, which is important for sexually reproducing

animals where sperm must survive in a different individual and compete for fertilization with other sperm (Zeng et al., 2013).

Materials and methods

Strains used

Targeting constructs were injected into the *EG6699: ttTi5605; unc-119(ed3)III* strain. To obtain males, the *him-5(e1490)* allele was present in all strains analyzed. Other alleles were *swm-1(me87), swm-1(me86),* and *swm-1(me66).*

Cloning single TIL domains into expression vectors

Generation of donor constructs and cloning into the PCFJ150 destination vector were performed as described in Chapter 2 Materials and methods. Primers used for generating constructs are listed in Table 3.1 and a description of plasmids used to generate targeting vectors is in Table 3.2

Quantification of sperm activation

Sperm activation was quantified as described in Chapter 2 Materials and methods.

Acknowledgments

I would like to thank Angela Snow for generating targeting vectors of single TIL domains.

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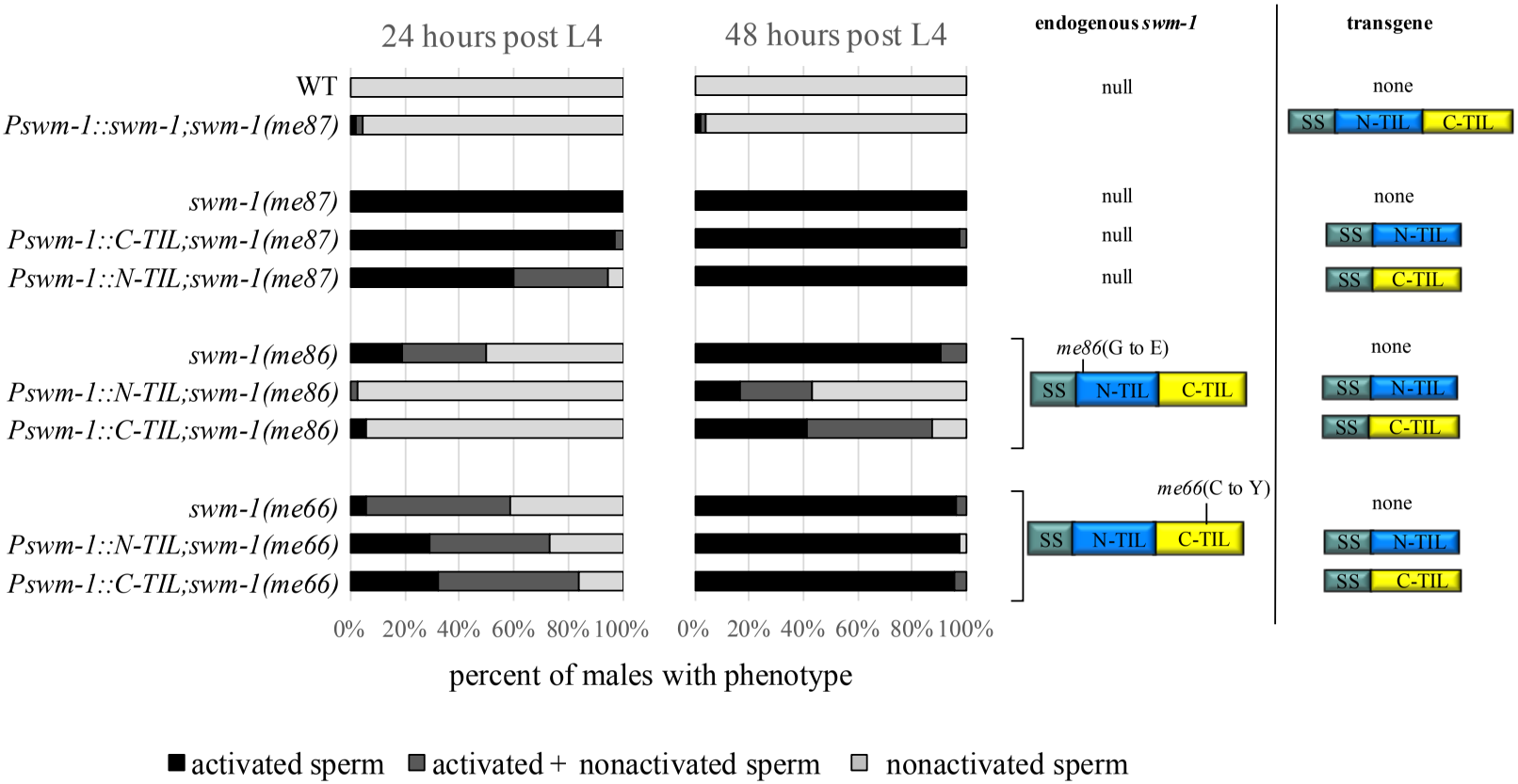

Figure 3.1 SWM-1 TIL domains play nonequivalent roles in sperm activation. Quantification of sperm activation phenotype of 24 hour and 48 hour post L4 single TIL domain adult males. Bars show the percentage of males with the indicated phenotypes. n = 24-50.

Table 3.1 Description of targeting vectors used to generate transgenic strains

Construct	Position 1 pDONR P4-P1r	Position 2 pDONR 221	Position 3 pDONR P2r-P3	Destination vector	Locus
Pswm-1::swm-1::swm-1 3'region	pAKS1	pAKS2	pAKS3	pCFJ150	ttTi5605
Pswm-1::swm-1::swm-1 3'region	pAKS1	pAKS2	pAKS3	pCFJ150	ttTi5605
Pswm-1::swm-1::swm-1 3'region	pAKS1	pAKS2	pAKS3	pCFJ150	ttTi5605

Table 3.2 Oligonucleotides used to generate targeting constructs

Fragment description	Fragment length	Forward Primer	Reverse Primer	Vector	Plasmid Name
swm-1 promoter	1365	GGGGACAACTTTGTATAGAAAAGTTGTGCCCATTTCTCACACAGAC	GGGGACTGCTTTTTTGTACAAACTTGTTTCACAATAACGAAGGGCAAC	pDONR™ P4-P1R	pAKS1
swm-1 coding region	630	GGGGACAAGTTTGGTACAAAAAAGCAGGCTTGAAAATGGTGAGTTTTTGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTATTTTGGACAATCTTTCTTATCAATG	pDONR 221	pAKS2
swm-1 N-TIL	450	GGGGACAAGTTTGGTACAAAAAAGCAGGCTTGAAAATGGTGAGTTTTTGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACTTGGTGCATTCAGATACTTC	pDONR 221	pAKS4
swm-1 C-TIL	343	GGGGACAAGTTTGGTACAAAAAAGCAGGCTTGAAAATGGTGAGTTTTTGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTATTATTTTGGACAATCTTTCTTATCAATG	pDONR P2R-P3	pAKS5
swm-1 3' region	729	GGGGACAGCTTTCTTGTACAAAGTGGTTTGTTTTTGAGTAAACATTTCCAAG	GGGGACAACTATGTATAATAAAGTTGACGCACAAATCCTTTCTTGC	pDONR P2R-P3	pAKS3

CHAPTER 4

SEQUENCING AND MAPPING OF *SWM-1* SUPPRESSOR MUTANTS

Introduction

The final step of *C. elegans* spermatogenesis, called sperm activation, is a dramatic cellular differentiation process involving remodeling of the cell in response to external signals. During activation, membrane protrusions dynamically spike from the spherical cell, cellular contents are reorganized, and specialized organelles called membranous organelles (MO) fuse with the plasma membrane (reviewed in L'Hernault, 2009). The resulting fully mature spermatozoon is a polarized cell with a pseudopod that it uses to crawl and to migrate towards eggs. In *C. elegans*, both males and hermaphrodites produce sperm, yet activation is regulated differently in each sex in terms of the location of activation and also the signaling molecules that regulate the process. An effort to understand *C. elegans* sperm activation at the molecular level has been the focus of several research groups and has revealed both novel and conserved aspects of reproductive biology, sperm biology, and cellular motility (reviewed in Ellis et al., 2014). In this chapter, I describe analysis of new genes important for sperm activation, including mapping and testing of candidate genes. Although this investigation was unsuccessful in identifying specific genes that regulate sperm activation, it generated whole-genome sequencing data for several mutants, produced candidate gene deletion alleles using

CRISPR, and useful information to consider for future work.

Activation of sperm by extracellular cues

Like the sperm of most species, the final steps of *C. elegans* spermatogenesis require extracellular signals. These are different in males and hermaphrodites. Within males, sperm remain nonactivated until they are transferred to the hermaphrodite during mating and become activated in response to seminal fluid. The seminal fluid activating factor is the serine protease TRY-5, which is produced in the vas deferens. The protease inhibitor SWM-1 is produced in somatic body wall muscle as well as in the vas deferens and is required to inhibit premature sperm activation in males (Smith and Stanfield, 2011; Stanfield and Villeneuve, 2006) (Chapter 2). While it is known that SWM-1 and TRY-5 are required for this regulation, the molecular mechanism by which SWM-1 and TRY-5 act are largely unknown. In hermaphrodites, sperm activates upon entrance to the sperm storage organ called the spermatheca. Hermaphrodites use a group of genes termed the *spe-8* group to regulate activation. Zinc is sufficient to activate sperm in a *spe-8*-dependent manner, although how zinc acts through *spe-8* group genes remains unknown (Liu et al., 2013).

The male and hermaphrodite sperm activation pathways are redundant; that is, the sperm from both sexes can respond to activators of the other sex, or undergo "trans-activation." This has been shown in two sets of experiments. First, *spe-8* mutant hermaphrodite fertility can be restored by mating to wild-type males (Argon and Ward, 1980). Second, *try-5* mutant male sperm can respond to the hermaphrodite activator (Smith and Stanfield, 2011). Together, these results indicate that there are two

redundant, separable modes of sperm activation. Studies of the sperm activation pathways have shown that the ancestral species of *C. elegans* had both the *swm-1/try-5* as well as the *spe-8* pathway (Wei et al., 2014), indicating the *C. elegans* species retained both modes of activation. *C. elegans* sperm also can be induced to activate *in vitro* with Pronase (a mixture of proteases), monensin (an ionophore), triethanolamine (TEA, a weak base), 4,4'-diisocyanatostilbene-2,2'-disulfonic acid (DIDS, a chloride channel inhibitor), calmodulin inhibitors, wortmannin, or zinc, although the mechanism by which these activators work is unknown (Bae et al., 2009; Machaca et al., 1996; Nelson and Ward, 1980; Shakes and Ward, 1989; Ward et al., 1983).

Sperm cell response to activation cues

On the surface of the sperm cell membrane, the solute carrier 6 family transmembrane protein SNF-10 is required for activation by TRY-5. *snf-10* is a component of the male pathway and *snf-10* mutant sperm can be transactivated in hermaphrodites (Fenker et al., 2014). In the hermaphrodite pathway, SPE-12, SPE-19 and SPE-29 are proteins that all have transmembrane domains and are predicted to act in a complex (Geldziler et al., 2005; Nance et al., 1999; Nance et al., 1999). Additional SPE-8 group genes include the nonreceptor tyrosine kinase gene *spe-8*, the novel protein *spe-27,* and *spe-47,* which encodes a protein predicted to interact with the sperm motility and signaling protein MSP (major sperm protein) (LaMunyon et al., 2015; Minniti et al., 1996; Muhlrad et al., 2014). Following receipt of the activation cue, downstream intracellular events proceed, including fusion of MOs with the plasma membrane and pseudopod formation (reviewed in Fraire-Zamora and Cardullo, 2010).

Identification of additional sperm activation factors

Although several components of each sperm activation pathway are known, the molecular mechanism by which they act to trigger differentiation is largely unknown. In the male, it is likely that SWM-1 and TRY-5 directly interact as SWM-1 is a serine protease inhibitor and TRY-5 is a serine protease. However, this has yet to be definitively shown. Furthermore, SWM-1 has two trypsin inhibitor-like domains and analysis of these domains suggests that it may have more than one target (Stanfield and Villeneuve, 2006) (Chapter 3). Therefore, it is possible that an unknown protease downstream of SWM-1 plays a role in the sperm activation pathway (Figure 4.1A). Another unknown in the male pathway is whether the sperm cell directly interacts with the TRY-5 protease. Indeed, it is unknown whether TRY-5 and SNF-10 directly interact and it is possible that another factor is required to transduce the TRY-5 signal (Figure 4.1A).

While *try-5* and *snf-10* were identified in a *swm-1* suppressor screen seeking to identify genes that suppressed the *swm-1* null phenotype, other factors could have been missed due to redundancy in the pathway. Therefore, a screen using the weak alleles of *swm-1* may uncover additional factors. This screen could yield factors downstream of *swm-1* that mediate transduction of the TRY-5 activation signal either upstream or downstream of *try-5,* other proteins on the sperm membrane that are important for receipt of the signal, or factors that interact with SNF-10 on the membrane. Furthermore, additional *spe-8*-class mutants could be identified in such a screen. Given what is known to activate sperm pharmacologically and that males depend on protease signaling, factors

that may be discovered include proteolysis regulators, secreted factors and membrane proteins.

Results

To identify other genes involved in sperm activation, a *swm-1* suppressor screen was performed by mutagenizing *swm-1(me86)* hermaphrodites with ethyl methanesulfonate (EMS). Individual F_1 hermaphrodites were placed on individual plates to lay progeny, and F_2 male progeny were scored for suppression of premature sperm activation. Strains homozygous for suppressor mutations were recovered from plates that showed at least 25% nonactivated males (Figure 4.1B). The screen resulted in 30 suppressor strains that suppressed premature sperm activation to varying degrees: 12 strong, 16 medium and 2 weak suppressors (Figure 4.1C). Complementation tests revealed that the strong suppressors were mainly mutations of *try-5* and *snf-10* but also included two new, unknown genes. Medium suppressors represented at least 4 unknown genes and the two weak suppressors were not analyzed (Figure 4.1C). Additionally, a *spe-8*-class mutant that showed hermaphrodite self sterility was identified.

Characterizing phenotypes of *swm-1* suppressor strains

The suppressor screen was performed over a period of 5 years and homozygous suppressor strains were stored at -80°C. Therefore, I re-scored sperm activation of the strongest candidates, which included those that were not *try-5* or *snf-10*, strong or medium suppressors, and those that did not complement each other (Figure 4.2A). I found that some strains that were initially characterized as strong or medium suppressors

were weak suppressors upon re-scoring. Therefore, I continued my analysis with the most consistently-strong suppressors, which included *jn6, jn8, jn12, jn14* and *jn19*. I tested whether the alleles were dominant or recessive and analyzed segregation frequency by analyzing F_1 and F_2 progeny (Figure 4.2B). I found that *jn6* and *jn14* were semi-dominant, as evidenced by non-activated F1 heterozygous males and more than 25% nonactivated F_2 males. The alleles *jn8*, *jn12*, and *jn19* were recessive, as evidenced by non-activated F_1 heterozygous males and no more than 25% non-activated F_2 males (Figure 4.2B). Lastly, I assessed whether suppressor strains were linked to *swm-1,* in which case I could look for variants near *swm-1* (see materials and methods). I found no evidence of linkage to *swm-1* for any of the suppressors (Table 4.1)

Mapping suppressor mutations

To map suppressor mutations, I used the single nucleotide polymorphism (SNP) mapping strategy developed by Davis et al. (2005). This strategy takes advantage of SNPs that differ between the standard laboratory strain, N2, and the closely related Hawaiian strain, CB4856, which has 1 SNP/~1000bp. SNPs can be detected using PCR and restriction digest detection assays, which facilitates mapping to a region of a chromosome by allowing recombination of the N2 and Hawaiian strain followed by pooling animals based on their mutant phenotype and genotyping SNPs (Figure 4.3) (Davis et al., 2005). I used this strategy to map *jn6* and *jn19*. A previous member of the lab, Amanda Mulia, mapped *jn15* to chromosome I between SNP markers 1,905,969 and 12,047,594 (Figure 4.4A). I mapped *jn19* to chromosome II between SNP markers 2,121,018 and 3,828,599 (Figure 4.4B) (Davis et al., 2005) . *jn6* appeared to be X-linked

(Figure 4.4C). I also attempted to map *jn8* but was unable to recover animals with the mutant, nonactivated phenotype. *jn14* samples were recovered and frozen.

The *jn12* strain was mapped with a similar strategy using recombination of the suppressor strain with the Hawaiian strain. However, rather than using PCR to detect SNPs, a larger sample of pooled DNA was collected for whole genome sequencing (WGS), followed by computational analysis of Hawaiian and N2 SNPs as described by Doitsidou et al. (2010) and Minevich et al. (2012). Following computational analysis using the Cloudmap software to detect SNPs, linkage of *jn12* failed to show clear linkage to a single genomic locus (Figure 4.5) (Minevich et al., 2012). Two regions of interest were notable: one on chromosome I and one on chromosome V (Figure 4.5A). The reduction in Hawaiian alleles on chromosome I corresponds to a known N2/Hawaiian incompatibility that causes embryonic lethality (Seidel et al., 2011; Seidel et al., 2008). The Cloudmap pipeline was used to account for known N2/Hawaiian incompatibilities, divergence of strains from their published reference genome, picking errors, and sequencing errors, as described by Minevich et al. (2014). After normalization, the chromosome I signal was largely absent (Figure 4.5B). The reduction in Hawaiian alleles on chromosome V was expected since the *swm-1(me86)* mutation was introduced into the Hawaiian strain by recombination and backcrossing and is also seen in the SNP chromosome mapping strategy (4.5B). It is interesting however that there were two predominant peaks, one between 8 - 8.5Mb and one between 9.5 -10Mb. The genetic and genomic position of *swm-1* likely corresponds to the smaller peak between 9.5 - 10Mb. Perhaps the second, largest peak between 8 - 8.5Mb warrants further investigation.

Identification of candidate genes

Following whole genome sequencing, reads were aligned to the reference genome and variants were called using the online Galaxy platform as described by Minevich et al. (2012). Variants were further filtered to include only protein coding and splice variants, homozygous mutations, lesions likely to be caused by EMS mutagenesis, inclusion of genes enriched in sperm and/or males, and mutations that were in common between mutants. For *jn15,* the strongest candidate was the *W09C3.2* gene, mainly because it was in the mapping region and predicted to be a membrane protein based on gene ontology (Table 4.2) (Ortiz et al., 2014; Reinke et al., 2000). The strongest candidate gene for *jn19* was *clec-130* (Table 4.3). *clec-130* is a c-type lectin predicted to have a carbohydrate binding function. Furthermore, lectins are a class of genes commonly found in various seminal fluid proteomics studies (de Lamirande et al., 1997; Ram and Wolfner, 2009).

Testing candidate genes for rescue of activation

To test the top candidate genes, I first attempted to rescue sterility of *jn15* mutants and activation suppression of *jn19* by introducing extrachromosomal arrays into the mutant strains. Arrays included 3' and 5' surrounding regions of *clec-130* (Figure 4.6A,B) and *W09C3.2* (Figure 4.6C,D). No rescue was observed in either case (Figure 4.6A-D). Although introducing extrachromosomal arrays did not rescue the phenotypes, I could not fully rule out the candidates because arrays can have variable expression and are rapidly be silenced in the germline. Furthermore, the *jn19* strain was very challenging to inject arrays into because the gonad of the hermaphrodite was very small and twisted.

As an alternative approach to thoroughly test the candidate activation genes, I introduced targeted deletions of candidate genes using CRISPR in the *swm-1(me86)* strain. I obtained two alleles of *W09C3.2* and one of *clec-130* that resulted in deletion of most of the gene in both candidate genes (Figure 4.7 A,B and Figure 4.8A). Neither deletion rescued the suppressor phenotypes (Figure 4.7 C and Figure 4.8B). I then generated CRISPR reagents to create deletions of other candidate genes in the mapping region, this time not excluding genes based on whether they were enriched in the male and/or germline. Isolation and testing of candidates beyond *W09C3.2* and *clec-130* was continued by another lab member, Abigail Greer. Genes for which deletion reagents were made and a summary of results can be found in Table 4.4.

Conclusions and future directions

Identification of additional sperm activation factors requires further investigation. In the case of *jn19,* a previous lab member attempted mapping using the SNP chromosome strategy and was unsuccessful due to no clear signal. Perhaps further analysis of *jn19* should include backcrossing to a nonmutagenized N2 strain to eliminate unwanted mutations. This process was initiated for *jn19* after the failure to identify the gene, and future investigation of this strain should be carried out with the backcrossed version. Furthermore, backcrossing should be considered for analysis of other suppressor strains, as many are sick and difficult to maintain. For example, the *jn19* strain had a very twisted gonad, which was seen in nearly every animal and made injecting constructs difficult.

In the case of *jn15*, the mapping region was very large, so identification of a gene may require additional mapping. A useful strategy may be to use the finer SNP mapping described by Davis et al. (2005). The linkage signal identified on chromosome V of the *jn12* strain is somewhat interesting due to its magnitude as compared to the signal closest to the genomic locus of *swm-1* (Figure 4.5B). Intriguingly, one variant near this region includes a male-enriched gene, *B0507.7,* that like SWM-1, is enriched in body wall muscle, coelomocytes and the germline (Ma et al., 2016; Spencer et al., 2011).

In conclusion, this work can serve as a starting point for future investigation of candidate sperm activation genes. One potential reason that I failed to identify additional genes may be functional redundancy of sperm activation genes. Sperm activation is necessary for fertility and thus it is reasonable to suspect there are genes with redundant function to ensure fertility and perhaps the fact that many *try-5* and *snf-10* alleles were uncovered suggests that suppression may require more than one gene.

Materials and methods

Quantification of sperm activation

Sperm activation of 48 hour post L4 adults was quantified as described in chapter 2 materials and methods. For strains carrying extrachromosomal arrays, animals were analyzed within 2 generations of array injection.

Linkage to *swm-1*

Candidate suppressor strains were crossed to the *mIs11; him-1(e1490)* strain and segregation frequencies for the activated and non-activated phenotypes were calculated to

determine whether the suppressor was closely linked to *swm-1*. Segregation of the nonactivated phenotype at a 3/16 ratio was considered unlinked to *swm-1*.

SNP chromosome mapping

To determine the genomic location of the *jn19* and *jn15* mutations, single nucleotide polymorphism (SNP) chromosome mapping was performed as described by Davis et al. (2005). To obtain samples for SNP mapping, *swm-1(me86) him-5(e1490); jn19* L4 males were placed with L4 hermaphrodites of the Hawaiian *swm-1(me86)him-5(e1490)* strain, a version of the CB4856 Hawaiian strain that had the closely-linked *me86* and *e1490* mutations introduced by recombination and backcrossing. After four days, to allow for mating and growth of cross progeny, 20 L4 hermaphrodites were placed on individual plates and allowed to lay eggs for two days, after which they were collected and PCR was performed to identify heterozygotes using the SNP_YD1 SNP assay from the Davis et al. (2005) primer set. From 6 different heterozygous plates, between 18 and 24 L4 males were picked and incubated at 20ºC for 48 hours. The sperm phenotype was scored by differential interference contrast microscopy as described in Chapter 2 and individual males were recovered and lysed in worm lysis buffer using standard protocols. Lysates were then pooled based on their phenotype, activated or nonactivated, and used for SNP chromosome mapping

SNP mapping by sequencing

Recombinant F_2 hermaphrodites segregating the suppressor phenotype were identified as described above and allowed to self-fertilize on 6cm plates until plates were

filled with adults. The progeny of 50 F_2 hermaphrodites were allowed to grow to the F_3/ F_4 generation, and starved for one day. Animals were collected and pooled into a 15mL conicle tube by washing plates with M9, spun for 2-3 min, rinsed three times with M9 and left for two hours at room temperature on a nutator to eliminate any remaining bacteria from the intestine. Worms were rinsed three more times with M9, spun down, remaining M9 was removed, and samples were frozen at -80°C. Genomic DNA was isolated using the Genomic DNA Extraction from Tissue kit (Thermo Fisher).

Whole-genome sequencing

DNA libraries were prepared for 150 bp paired-end sequencing by the University of Utah sequencing core and subjected to whole genome sequencing on an Illumina MiSeq sequencing platform.

Transgenic strains

To generate strains carrying extrachromosomal arrays, *clec-130* and *W09C3.2* coding sequences that included 3' and 5' surrounding region were amplified from genomic DNA and injected into the mutant strains (Table 4.5).

Candidate gene deletions

To generate deletions in candidate genes, guide RNAs were designed using the benchling.com CRISPR design tool, which uses algorithms to identify and calculate gRNA efficiency and off-target scores as published by Hsu et al. (Table 4.6) (Hsu et al., 2013). gRNA sequences were cloned into the pDD162 plasmid as described in

Dickinson et al. (2013) and injected with the *rol-6* (pRF4) and *unc-22* (PCCM935) co-CRISPR plasmids into N2 hermaphrodite gonads (Dickinson et al., 2013; Kim et al., 2014). Deletions were detected by PCR amplification of regions flanking the targeted site in F1 hermaphrodites.

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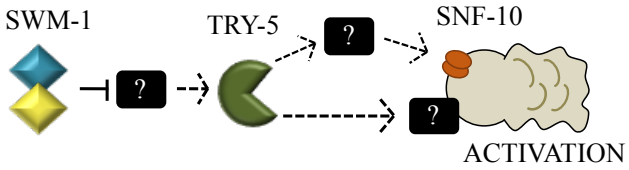
A



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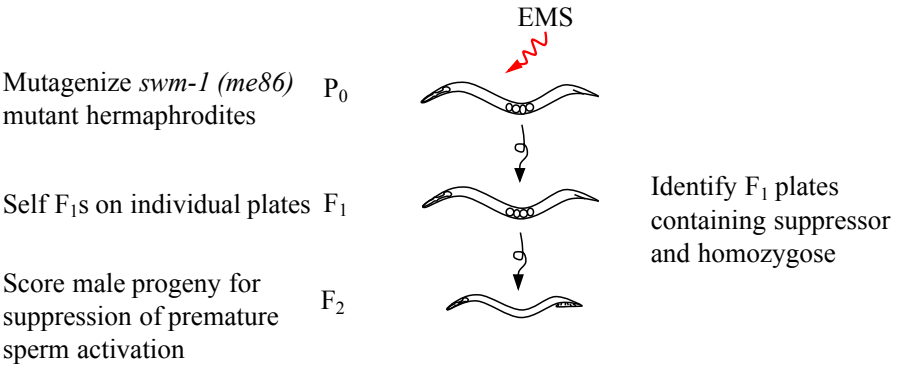


C

Degree of *swm-1* suppression	# of alleles	Genes
strong (>75%)	12	*try-5* (5), *snf-10* (5), new (2)
medium (40-75%)	16	at least 4 unknown
weak (<40%)	2	not analyzed

Figure 4.1 Summary of suppressor screen. A) Schematic of known sperm activation pathway genes including potential interacting factors that could be uncovered in a *swm-1* suppressor screen. B) Summary of *swm-1* suppressor screen. C) Summary of alleles recovered from the *swm-1* suppressor screen.

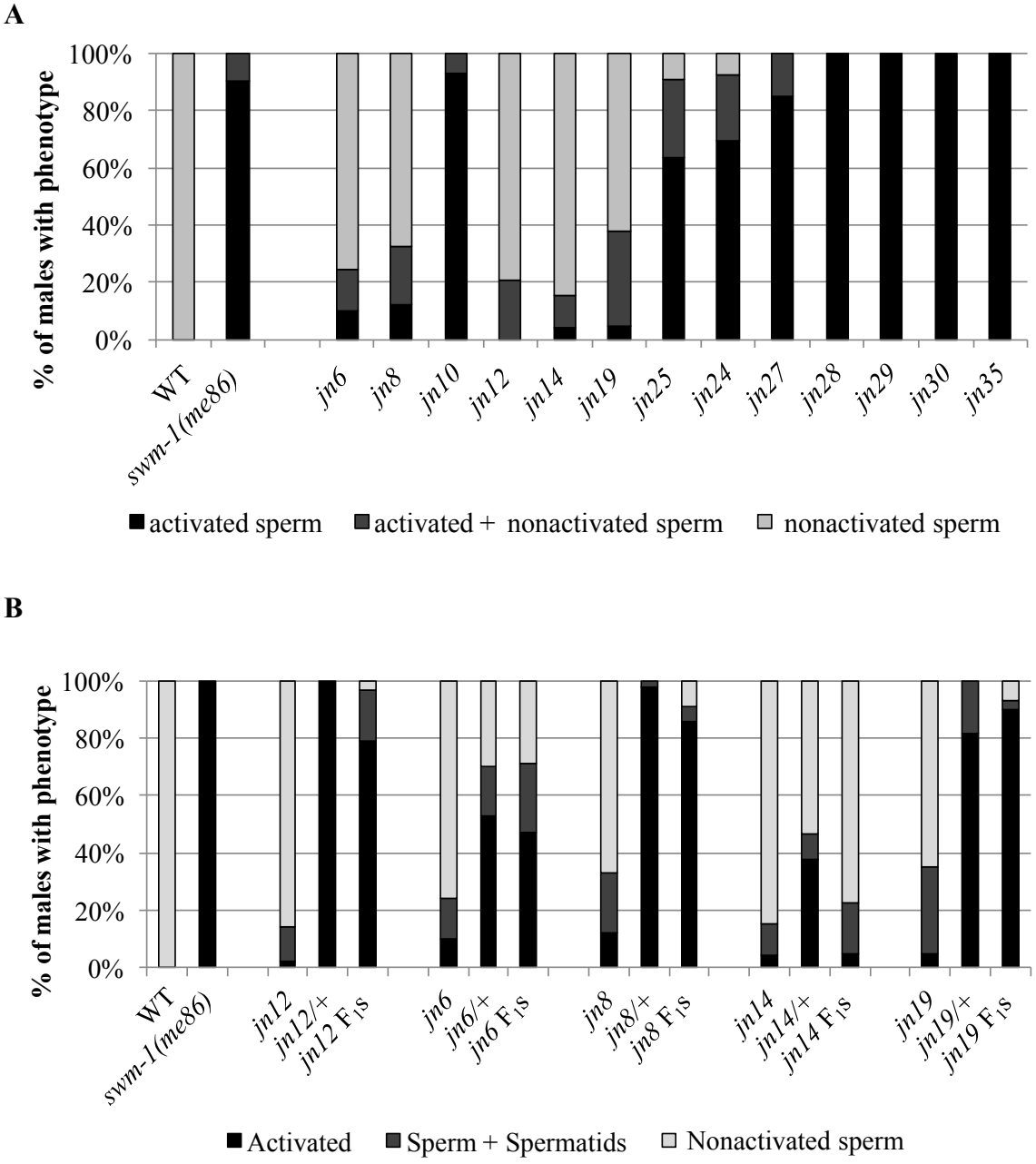


Figure 4.2 Strains isolated from swm-1 suppressor screen suppress premature activation of *swm-1(me86)* mutants. A) Quantification of sperm activation of *swm-1* suppressors in 48 hr post L4 adult virgin males. B) Quantification of segregation frequency of suppressor phenotypes. Males contain either nonactivated (light grey), a mix of sperm and spermatids (grey), or activated sperm (black). Phenotype of strains originally isolated with >40% of males containing nonactivated sperm. Alleles *jn10, jn25, jn24, jn27, jn28, jn29, jn30* and *jn35* show elevated levels of activation.

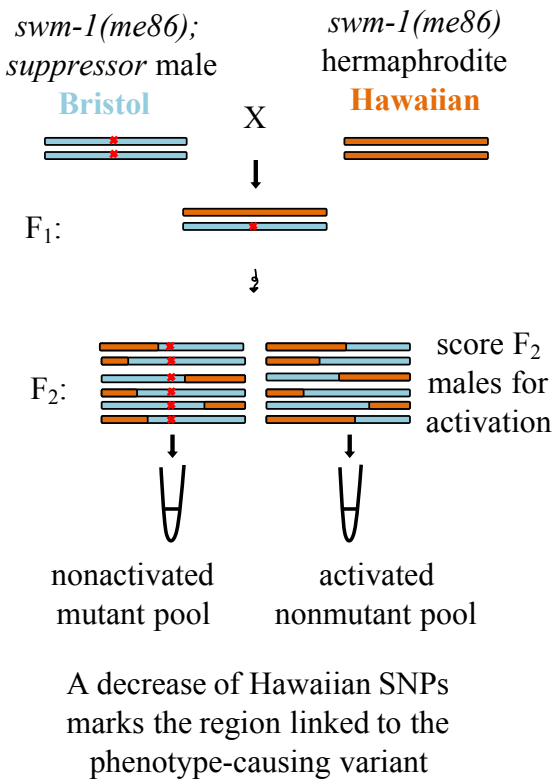
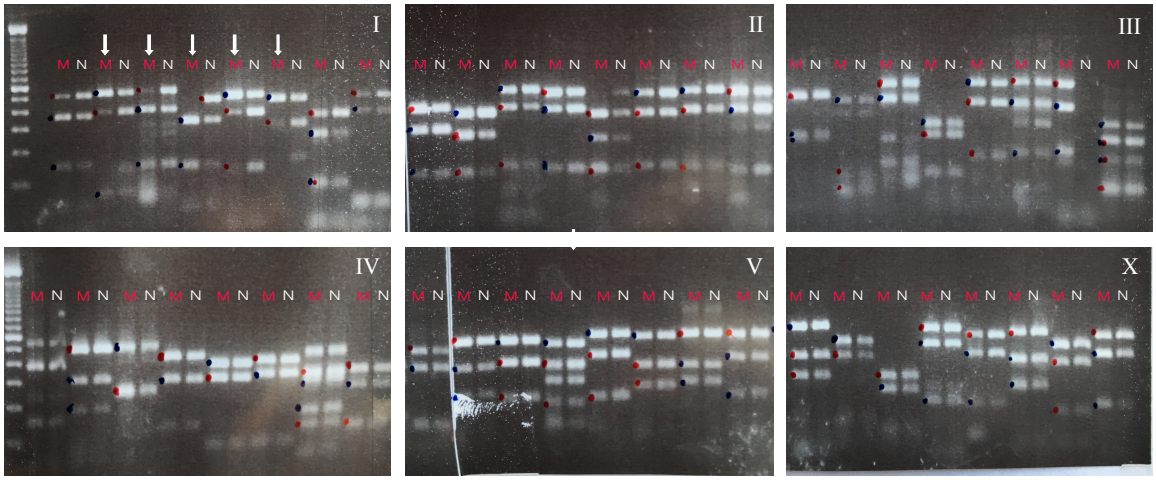


Figure 4.3 Schematic of SNP mapping strategy. Suppressor males are crossed to Hawaiian *swm-1(me86)* hermaphrodites. Heterozygous hermaphrodites are identified by PCR and F_1s are allowed to self-fertilize. F_2 males are scored for their activation phenotype, pooled, and prepared for either SNP chromosome mapping or mapping by sequencing.

Figure 4.4 Chromosome mapping of *jn15*, *jn19,* and *jn6* suppressor strains. Agarose gel images of *jn15* and *jn19* chromosome mapping results. Arrows show regions of suppressor allele linkage to a chromosomal location where there is a decrease in Hawaiian SNPs. Each pair represents SNP assay results from mutant recombinants (M) followed by a non-mutant (N) recombinants. Blue dots indicate N2 bands, red dots indicate Hawaiian bands. A) *jn15* mapping. *jn15* is linked to chromosome I between SNP markers at 1,905,969 and 12,047,594. B) *jn19* is linked to chromosome II between SNP markers at 2,121,018 and 3,828,599. C) *jn6* is linked to the X chromosome.

A



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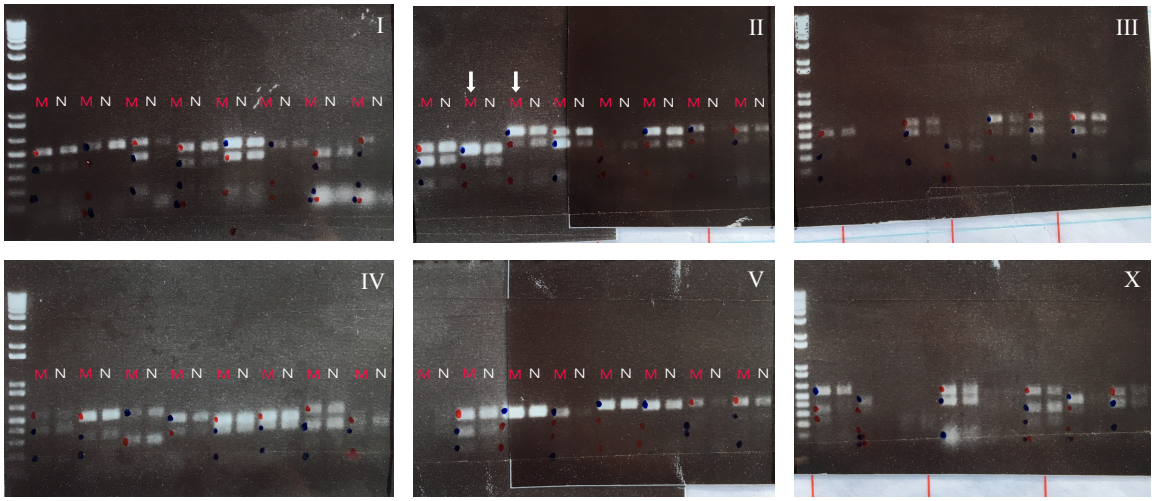

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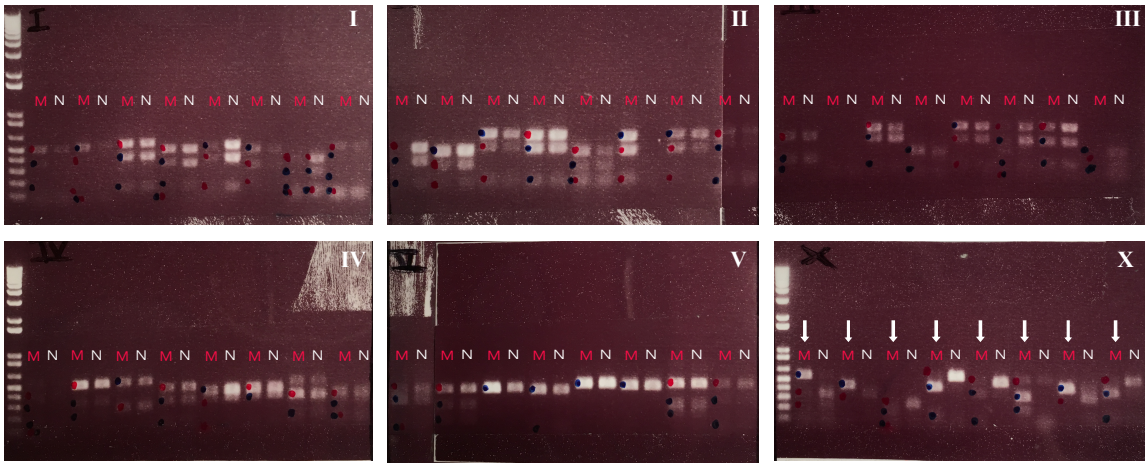


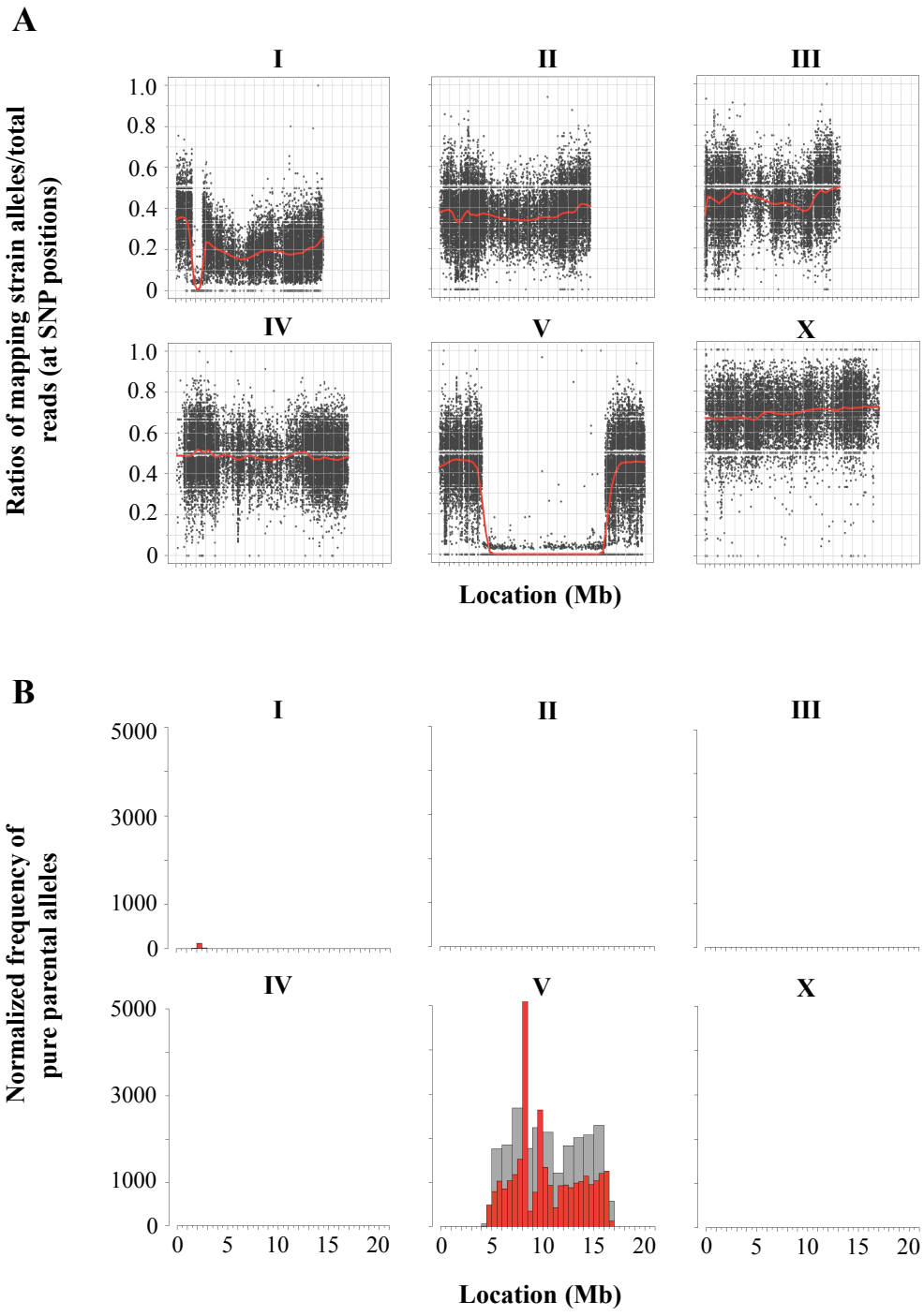
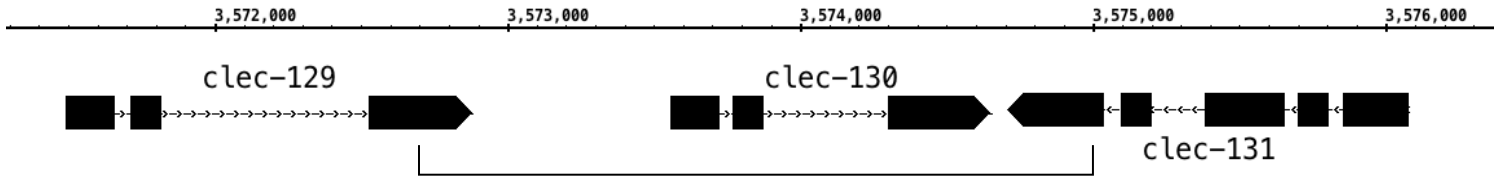


Figure 4.5 Cloudmap analysis of SNP mapping by sequencing *jn12*. A) Graphs of SNP ratios across each chromosome. Red line indicates LOSSES regression line (see Minevich et al. 2014). Chromosome I shows a decrease in Hawaiian alleles at genomic position ~1.5Mb that corresponds to a known N2/Hawaiian incompatibility. Chromosome V shows a decrease in Hawaiian alleles between genomic positions 5-15Mb. B) Normalized parental allele frequency.

Figure 4.6 Extrachromosomal arrays of *jn15* and *jn19* candidate genes. A) Schematic of *clec-130* region amplified for testing array rescue of *jn19* mutants. B) Quantification of sperm activation phenotype of *jn19* mutants carrying extrachromosomal arrays of the *clec-130* gene and regulatory sequence. Five independent array strains showed no rescue of the mutant phenotype. Black indicates males that contain activated sperm, grey indicates males that contain a mix of sperm and spermatids, light grey indicates males that contain nonactivated sperm. C) schematic of *W09C3.2* region amplified for testing array rescue of *jn15* mutants. B) Quantification of hermaphrodite sterility phenotype of *jn15* mutants carrying extrachromosomal arrays of the *W09C3.2* gene and regulatory sequence. 35 individual F_1 hermaphrodites carrying arrays showed no rescue of sterility phenotype.

A

II


3,572,000
3,573,000
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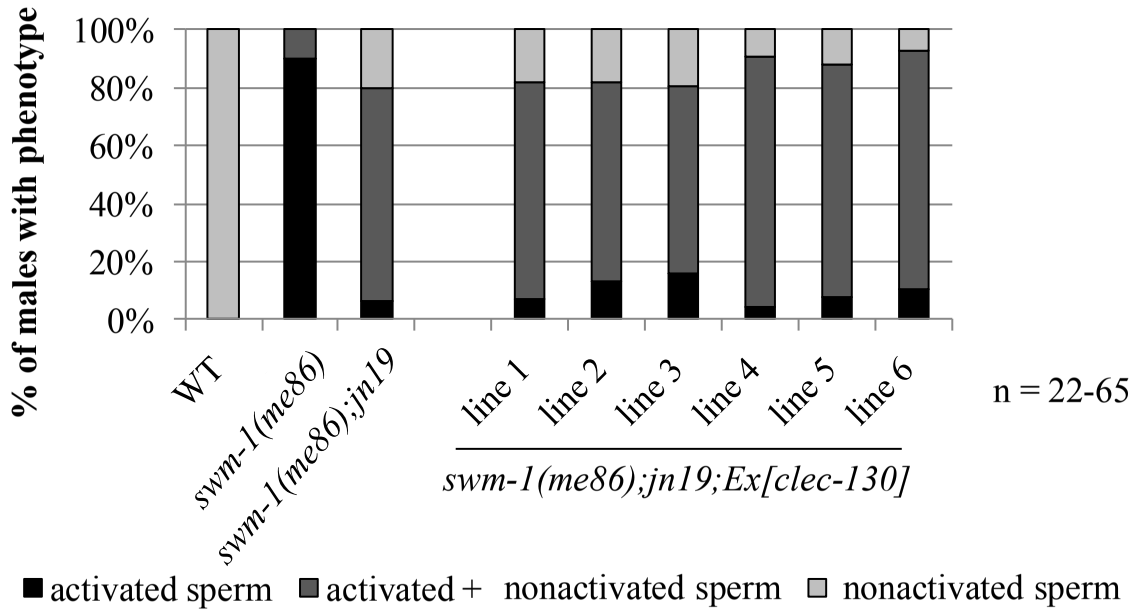
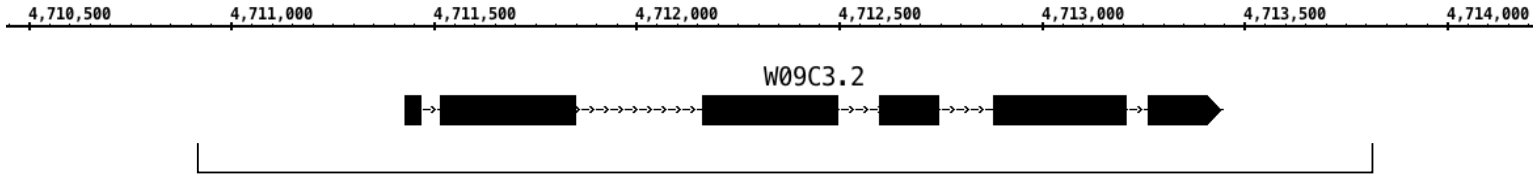
clec-129
clec-130
clec-131

B


% of males with phenotype
100%
80%
60%
40%
20%
0%
WT
swm-1(me86)
swm-1(me86);jn19
line 1
line 2
line 3
line 4
line 5
line 6
swm-1(me86);jn19;Ex[clec-130]
n = 22-65
activated sperm
activated + nonactivated sperm
nonactivated sperm

C

Chromosome I

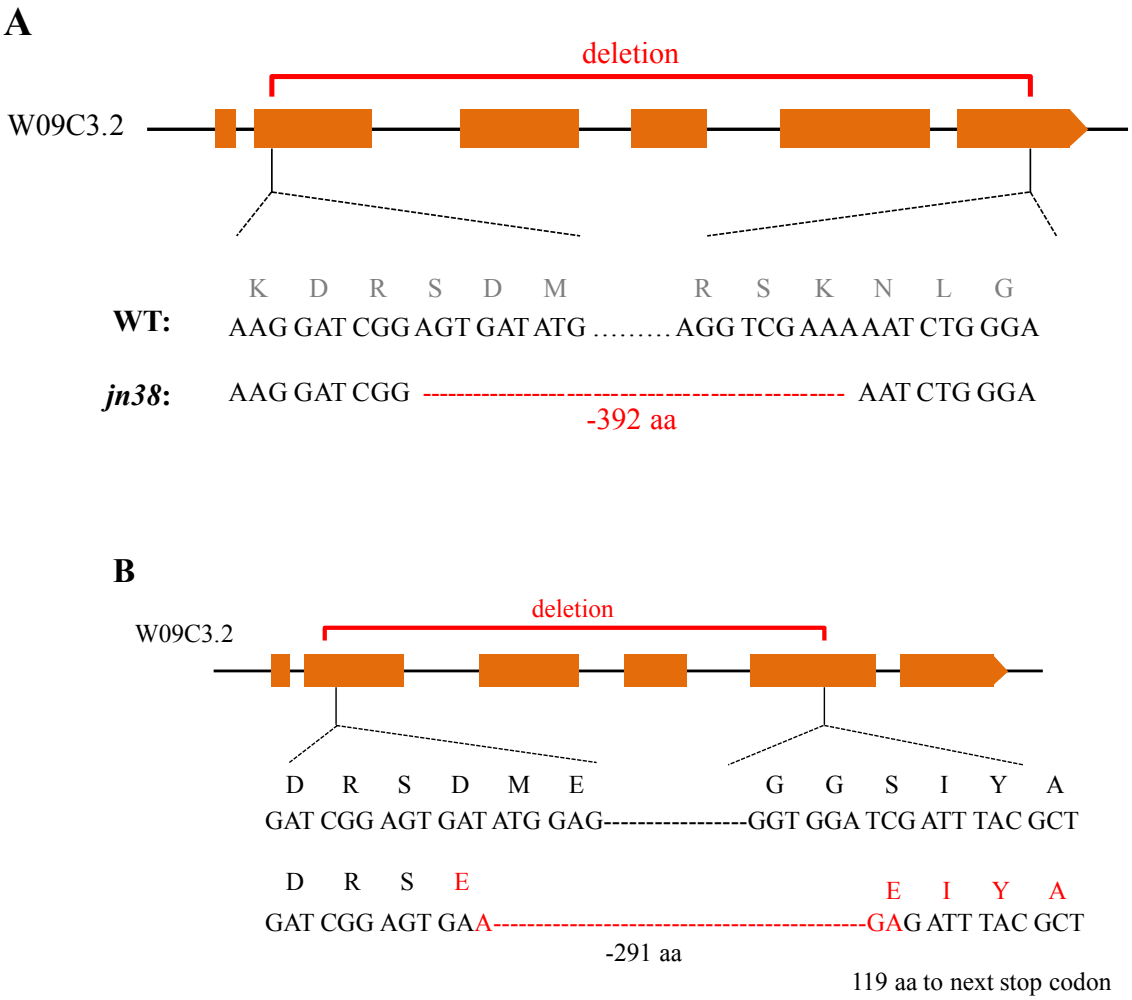


D

Hermaphrodite fertility

genotype	% sterile	n
swm-1(me86);jn15	100	20
swm-1(me86),jn15; Ex[W09C2.3]	100	35

Figure 4.6 continued



C

genotype	% sterile	n
swm-1(me86);jn15	100	20
W09C3.2(jn38)	0	4 (independent lines)
W09C3.2(jn38); swm-1(me86)	0	3 (independent lines)

Figure 4.7 CRISPR deletion alleles of W09C3.2 A,B) Schematics of *W09C3.2* deletion alleles. C) *jn15* hermaphrodite sterility is not rescued by *W09C3.2* deletions.

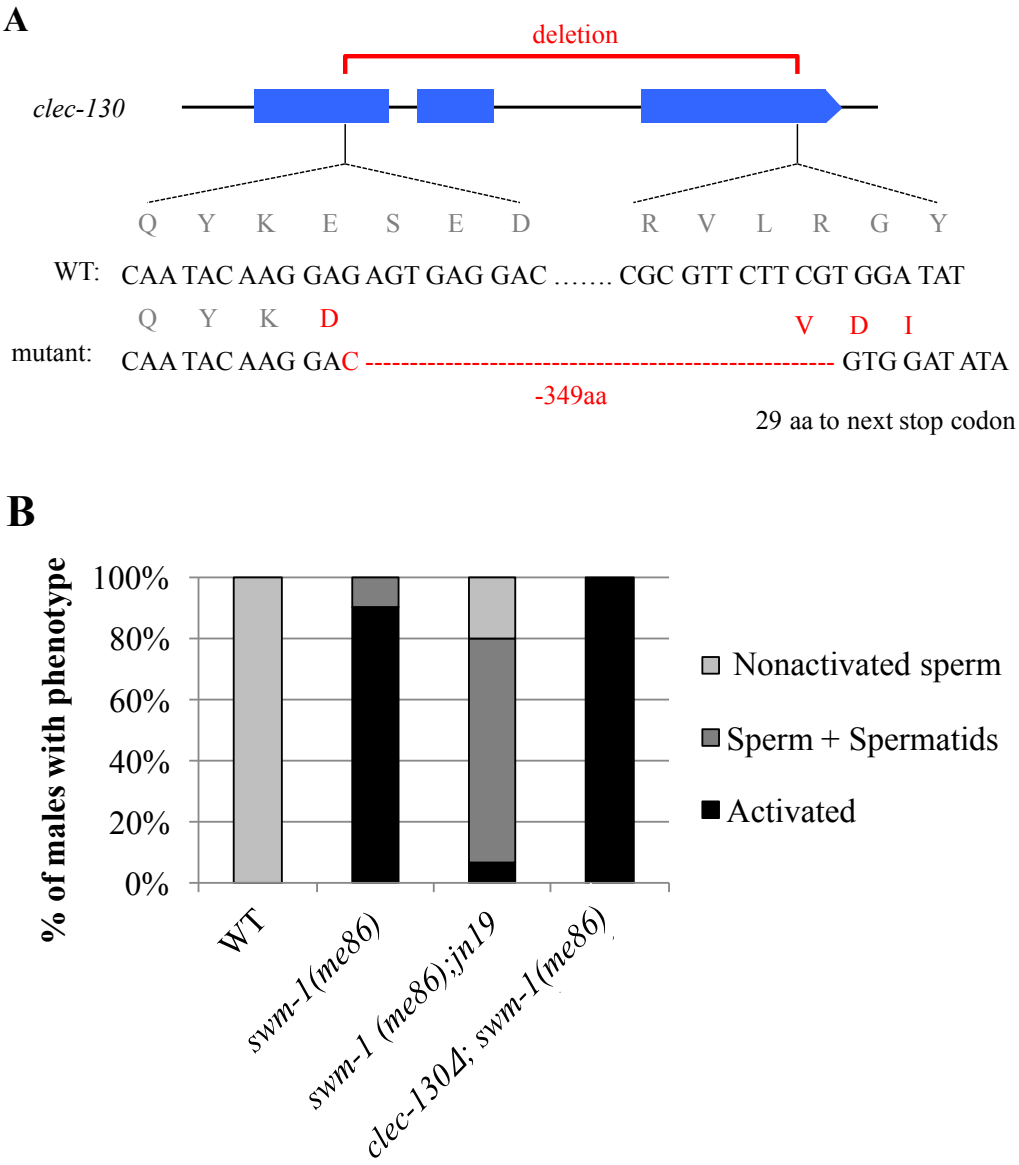


Figure 4.8 CRISPR deletion of *clec-130*. A) Schematic of the *clec-130* deletion allele. B) Quantification of the sperm activation phenotype of the *clec-130* deletion allele. Deletion mutants show no suppression of *swm-1(me86)* activation phenotype.

Table 4.1 *swm-1* linkage analysis.

suppressor strain	F2 phenotype (Activated – sperm and spermatids – nonactivated%)	linked to *swm-1?*
jn6	10 – 16 – 74	no
jn8	16 – 3 – 81	no
jn12	16 – 15 – 58	no
jn19	22 – 16 – 74	no

Table 4.2 Summary of *jn15* candidate genes in mapping region

Position	Gene	Reference	Change	Amino acid change	Coverage	predicted function/ conserved domain
1142104	*Y48G8AL.10*	C	A	G/C	21	nucleic acid binding, metal ion binding
2379644	*sydn-1*	C	T	M/I	29	neuron development
4124545	*H31G24.1*	G	A	G/R	33	embryo development
4711711	*W09C3.2*	G	A	S/N	31	splicing
4765803	*M04F3.2*	G	T	STOP	3	unknown
7070493	*tofu-5*	C	T	R/C	31	embryo development and reproduction
7301404	*ttx-7*	C	G	G/A	39	inositol biosynthesis
8817033	*F36A2.9*	C	T	P/L	29	Lipase, GDSL, SGNH hydrolase-type esterase domain
9246867	*F36H2.2*	G	C	R/G	35	membrane nucleoside transporter
9628158	*hsr-9*	C	T	S/F	24	BRCT- cell cycle regulation
10888886	*lrk-1*	C	T	G/D	37	Ankyrin repeat, kinase

Table 4.3 Summary of *jn19* candidate genes in mapping region

Position	Gene	Reference	Change	Amino acid change	Coverage	predicted function/ conserved domain
2757933	*F29A7.8*	C	T	G->R	23	unknown
2800568	*nhr-119*	G	A	S->L	19	DNA-binding, transcription factor
3573598	*clec-130*	G	A	G->S	31	carbohydrate-binding
3797068	*Y8A9A.2*	C	T	P->L	88	Thrombospondin, type 1 repeat

Table 4.4 Additional candidate gene alleles tested

suppressor	candidate gene	allele	*swm-1(me86)* suppression (% of males with nonactivated sperm)
jn19	*clec-130*	*jn54*	0% (n=31)
jn19	*clec-130*	*jn55*	0% (n=23)
jn19	*clec130*	*jn56*	no data
jn19	*clec-130*	*jn57*	no data
jn19	*F29A7.8*	Δ1	0% (n=52)
jn19	*nhr-119*	*jn53*	0% (n=54)
jn15	*F36A2.9*	none obtained	no data
jn15	*tofu-5*	none obtained	no data

Table 4.5 List of primers for extrachromosomal arrays and detecting deletions

Fragment description	Fragment length	Forward primer	Reverse primer
clec-130 deletion detection	1284 (WT)	5'-GCACATTGC ATCGAGCTG	5'-GTGAACTGTG TCGGAAACTTTG
clec-130 array rescue	2275	5'-CCAGGGCA ATCAACCAGAC	5'-GAATCAGACAATG GCAGCC
clec-130 array rescue (for nested PCR)	2364	5'-GCTTTCGAG TACACCGACG	5'-CTCGATATGGGT TGGCGTTC
F29A7.8 deletion detection	775 (WT)	5'-TCCATTGAGCT CACGGTTTC	5'-CGGATACCTAA TTCAGTTCC
nhr-119 deletion detection	5554 (WT)	5'-TTAAGCCAAAT CGCGTCAC	5'-CACCTAGAAATG GCCAGAAAATC
W09C3.2 array rescue	3055	5'-CGCGAGGT CAATTACACGC	5'-GTTGGACGAGT AGAGGTGG
W09C3.2 array rescue (for nested PCR)	3284	5'-CCAAAGTTGTC GTCCGTC	5'-TCCGCGTTCCA GATTCATTTC
W09C3.2 deletion detection	2542 (WT)	5'-GGATCTTTA GTGCCTGGGAAG	5'-GAAATGAATC TGGAACGCGGA
F36A2.9 deletion detection	2635 (WT)	5'-TTTAGCCTT ATCGCAAGCAC	5'-GCTTCTTGAT GGCAAGGTAG
tofu-5 deletion detection	1127 (WT)	5'-ACGGCGATCCC ACAAATG	5'-ACGATGTAGA TGGCTGGTTG

Table 4.6 List of guide RNAs used to target Cas9 to candidate suppressor genes

gene	guide RNA #	target sequence (PAM)
clec-130	3	5'-CAACGTCGCTGCAAAATATT(GGT)
clec-130	4	5'-ATGCGGCACGCGTTCTTCGT(GGA)
clec-130	5	5'-TCCCAATACAAGGAGAGTGAGGACAAT(GGA)
clec-130	6	5'-TCCTTTAATCGGCCTTCTGGA(GGA)
F29A7.8	1	5'-TAAAAATGTTAAAAATGTG(GGG)
F29A7.8	2	5'-AAGCAGTCACTCATACAGTC(GGT)
F29A7.8	4	5'-TTCTAGCTTCTACTGGCGGA(GGA)
nhr-119	1	5'-TACAAGTGTCACTGCCGCCT(GGA)
nhr-119	2	5'-GGTGAGGTATCCATACCGGA(GGT)
nhr-119	3	5'-CACACAGCTACTAGCTAGGC(GGA)
W09C3.2	3	5'-CTGCAATTCTAATATCAAT(TGG)
W09C3.2	4	5'-CACAAGGATCGGAGTGATAT(GGA)
W09C3.2	5	5'-GAGTTTTCTTAGATGGAGAT(GGA)
W09C3.2	6	5'-AAAGGAGAAAGCTAAAAAG(AGG)
F36A2.9	1	5'-GACCAGAAAATTCTACGCGG(GGA)
F36A2.9	2	5'-TATGGAATAGGCTGATTGGC(GGA)
tofu-5	1	5'-GAAAACTGACTCTGTCCAGT(GGT)
tofu-5	2	5'-TATCCTCCGAACGTTATGGC(GGC)

CHAPTER 5

SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS

Summary

In the work presented in this dissertation, I discovered that *C. elegans* males use a surprising cell type, muscle cells, to generate the extracellular environment within the gonad that ensures male fertility. This work lead to extended studies on soma-germ line communication where I showed that other proteins behave this way and that this phenomenon has implications for future studies of *C. elegans* male fertility. This work also led to the surprising discovery that SWM-1 is a seminal fluid protein and is present in the sperm migratory environment, though its role in the hermaphrodite remains unknown. I tested models of how SWM-1 may be interacting with its likely target, TRY-5, to demonstrate that they do not regulate localization of each other, a step towards understanding the molecular mechanism by which they act (Chapter 2). I carried out investigation of the molecular protein domains of SWM-1, work that provided further evidence that SWM-1 may be a multifunctional protein (Chapter 3). I went on to begin initial characterization of mutant strains that suppress the premature activation of *swm-1* mutants (Chapter 4). Lastly, since I found that SWM-1 is present in the sperm migratory environment and found additional evidence that SWM-1 has multiple targets, I

investigated the possibility that it can affect sperm migration and competition between sperm of multiple males (Appendix).

Extended discussions and future directions

Soma-germ line interactions: considerations for *C. elegans* and beyond

The discovery that muscle is the critical source of the SWM-1 inhibitory signal is undoubtedly a curious and unexpected finding (Chapter 2). It represents, to my knowledge, the first example of muscle being an important regulator of postmeiotic sperm maturation. Why would regulating sperm motility rely on secretions from muscle? In the discussion in Chapter 2, I propose this tissue source can provide a persistent signal of inhibitor as opposed to somatic gonadal cells, which are less numerous and where secretion products are continually depleted during ejaculation. In mammals, smooth muscle surrounds most tissues of the male reproductive tract, including the testis, epididymis and vas deferens. It is known primarily for its structural role, providing peristaltic movement of sperm and seminal fluid and for androgen signaling that is important for epithelial function and identity (Belleannée et al., 2012; Welsh et al., 2010). No sperm maturation factors are known to be secreted from this muscle in any other organism. Yet given the mounting evidence that muscle is an important endocrine organ, and that it is a key regulator in the stem cell niche in the *drosophila* intestine, contributions from muscle remain possible and may be important considerations for future studies (Iizuka et al., 2014; Lin et al., 2008; Pedersen, 2013; Pedersen and Febbraio, 2012; Pedersen, 2013).

Future research in *C. elegans* may seek to determine the mechanism by which systemic factors in the pseudocoelomic cavity enter the seminal vesicle. It is known that cells that compose the seminal vesicle express the gap junction proteins innexin 8 and innexin 9, and that these are important for germline proliferation and gametogenesis (Starich et al., 2001; Starich et al., 2014). Presumably, these form channels much too small for a large protein such as mCherry or GFP to pass. Indeed, I tested this idea and found that *inx-9 inx-8* double mutant animals do not display premature sperm activation and that SWM-1::mCherry is present in the seminal vesicle of these mutants (data not shown). These gap junction proteins are the only channels known to regulate soma-germ line interactions in *C. elegans* but my data suggest that there is another means by which factors are exchanged. One way to study this may be by analysis of the movement of injected dyes or other molecules with known sizes and biochemical properties into the pseudocoelomic cavity. This technique has been demonstrated in the hermaphrodite but may be adapted in males (Fares and Greenwald, 2001).

C. elegans sperm activation

One of the challenges to understanding the mechanisms of sperm activation lies in the fact that there are very likely unknown components of the pathway. As I discuss in Chapter 4, this may be due to functional redundancy and may require multiple gene mutations to uncover a phenotype. My finding that muscle is an important source of secreted factors that regulate sperm may aid in filtering variants identified in sequencing data, as such factors need not be limited to genes showing expression in the germline or gonad. Perhaps additional filters using data from transcriptomic studies and

bioinformatic analysis of *C. elegans* genes can be applied to candidate gene lists. These studies have identified putative secreted proteins in the *C. elegans* genome as well as a muscle transcriptome (Hutter and Suh, 2012; Ma et al., 2016). Indeed, the fusion PCR method of generating guide RNAs to target Cas9 vastly facilitates rapid generation of deletion mutants to test candidates and should be used to generate deletions using CRISPR in future work (Ward, 2015). Additionally, it may be interesting to analyze various known *unc* mutants, which display an uncoordinated phenotype. Many such mutants have been described, and several affect body wall muscle. Therefore, it may be interesting to analyze SWM-1 secretion and the sperm of these mutants (Gieseler et al., 2017).

My investigation of the molecular mechanism by which SWM-1 and TRY-5 interact included analysis of the co-localization of SWM-1 and TRY-5 in males and hermaphrodites and using *spe-6* mutants to test whether localization of each protein was dependent on the other. These results support a model where SWM-1 directly inhibits TRY-5 but further investigation including *in vitro* biochemical approaches will be required to determine the mechanism of action of SWM-1 and TRY-5.

SWM-1 as a component of the sperm migratory environment

Although my investigation did not uncover a clear role for SWM-1 in the hermaphrodite uterus, localization of SWM-1 to the uterus suggests that it is important for some aspect of sperm success. In my experiments, I tested whether vas deferens-derived SWM-1 was important for sperm migration and/or male fertility. Although I did not uncover a strong effect on male fertility, one caveat to my experiments may be the

inability to precisely modulate levels of SWM-1 using the tissue-specific promoters. Specifically, the body-wall promoter to generate "vas-" males does not account for the possibility that this promoter likely generates higher levels of SWM-1 in the seminal vesicle, which could compensate for SWM-1 absent from the vas deferens.

SWM-1 is not required for hermaphrodite self-fertility yet it is expressed in the hermaphrodite, and, in a sensitized genetic background, its loss improves activation of self sperm (Stanfield and Villeneuve, 2006). Furthermore, as mentioned in the discussion in Chapter 2, SWM-1 in the uterus may be important in less than ideal conditions or to provide a small benefit to reproductive success such as during sperm competition. Although I performed sperm competition experiments with *swm-1* mutant males, control experiments did not support the experimental outcome and should be repeated. Additionally, these experiments may have suffered from a lack of sensitivity. Therefore, a more sensitive assay may aid in identification of a phenotype. In my experiments, sperm success was measured as total progeny sired by a male, a relatively crude measure of sperm success. Perhaps a role may be uncovered by a simplified experiment such as beginning by testing male precedence over hermaphrodite sperm as a starting point and to provide a simplified experimental paradigm. Using total male fertility as a readout is further complicated by the significant variability between males in terms of mating frequency, and number of sperm and or seminal fluid transferred. Therefore, perhaps a shorter window of mating time and verifying efficient mating may assist in identification of a phenotype.

C. elegans males as a model of male reproductive tract and secretory biology

The cell biology of the male reproductive tract in *C. elegans* is poorly studied yet represents an opportunity to research secretory and male reproductive biology in a transparent tissue. Through my investigation of the role of SWM-1 in sperm activation, I performed tissue-specific expression of SWM-1. Notably, as mentioned in the discussion in Chapter 2, expression of secreted proteins in cells of the vas deferens, the valve and cuboidal cells, largely resulted in sequestration of proteins. By contrast, neurons, epidermis, and muscle seem to secrete the same proteins, continually, without sequestration in vesicles like in the vas deferens. Within vas deferens cuboidal cells, SWM-1 exhibits a distinct subcellular localization pattern. In cuboidal cells, SWM-1 (and TRY-5) are contained in numerous intracellular membrane-bound vesicles of various sizes. The morphology and function of these cells is reminiscent of *Drosophila* accessory gland secondary cells which secrete exosomes, and seminal fluid (Corrigan et al., 2014; Sitnik et al., 2016). The largest vesicles are closest to the apical membrane, presumably poised for rapid secretion during ejaculation, and the nucleus is located basally. The size of the large vesicles is striking, some reaching up to 10μm in diameter and I often visualized what appeared to be smaller vesicles within these large vesicles. (data not shown). A few electron micrographs of the vas deferens have been published (Lints and Hall, 2009). These show some secretory globules near the apical membrane as I have observed. However, these micrographs likely represent only one type of vas deferens cell, though at least three have been described. Recent electron micrographs that have yet to be analyzed are likely to better characterize the nature of these vesicles (Kristin Fenker and Gillian Stanfield, University of Utah, personal communication).

One of the biggest challenges in understanding the role of seminal fluid and sperm maturation is that the tissues are difficult to obtain and must be fixed for analysis. *C. elegans* provides a unique opportunity to visualize seminal fluid production, secretion and function. However, SWM-1 is only the third *bona fide* seminal fluid protein in *C. elegans* shown to be transferred to the hermaphrodite uterus during mating. Although there is not a complete list of known seminal fluid proteins for *C. elegans*, several have been shown to be expressed in the vas deferens, have a secretion signal and represent the classes of proteins commonly found in the seminal fluid of most species (Thoemke et al., 2005). Furthermore, with the extensive transcriptomic analysis that has been reported on secreted and male-enriched transcripts and proteins in *C. elegans*, a candidate list of factors is feasible to generate (Ortiz et al., 2014; Reinke et al., 2000; Reinke et al., 2004).

Future studies on seminal fluid production, secretion and secretory vesicle dynamics should take advantage of abundant markers that are available for these structures. I have generated strains that have the intracellular vesicles of valve cells and cuboidal cells marked with a GFP membrane marker as well as secreted mCherry-marked seminal fluid in both valve and cuboidal cells (data not shown). Interestingly, in my initial analysis of these strains, it seems that there is likely a distinct cell type of valve cells that does not express either of these markers. This further highlights the utility of this system which is simplified but resembles the tubular structures of the male reproductive system of other organisms where there are multiple cell types that together orchestrate the requirements of sperm success.

Overall, the work presented here has uncovered novel cell biology and opened many other avenues of research that can be pursued using the *C. elegans* male. My hope

is for future studies to investigate the role of SWM-1 in the sperm migratory path, SWM-1 TIL domain function, identification of other sperm activation genes and studies to identify other seminal fluid proteins and lastly, investigation of the fascinating membrane biology of the *C. elegans* vas deferens.

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APPENDIX

ANALYSIS OF SPERM MIGRATIO AND COMPETITION IN *SWM-1* MUTANTS

Introduction

Since I showed that SWM-1 is a seminal fluid protein contributed to the uterus during mating and that hermaphrodites also produce SWM-1 that is present in the uterus, I hypothesized that it can affect some aspect of postmating sperm success. I decided to test if SWM-1 could affect sperm migration and competition because it does not affect hermaphrodite self-fertility and SWM-1 in male seminal fluid is not critical for male fertility. This lead me to hypothesize that it has a different role in the uterus aside from its known role as an inhibitor of activation (Stanfield and Villeneuve, 2006) (Chapter 2). Interestingly, overexpression of SWM-1 in seminal fluid reduces male fertility, an effect that can be relieved by removing SWM-1 in hermaphrodites (Chapter 2). This suggests that the levels of SWM-1 in the uterus are optimized for sperm success. However, these experiments show that SWM-1 can affect sperm and do not address whether these affects are due to SWM-1s normal role in sperm success or if there is toxicity by the overexpression. Thus, its presence and role in the uterus remains mysterious. Despite the fact that it is not critical for fertility, it is nevertheless supplied by both males and hermaphrodites to the uterus, implicating that it has an important function, perhaps to optimize some aspect of gamete success. Intriguingly, the *drosophila* seminal fluid

protein Acp62F is also a TIL domain serine protease inhibitor present in the female spermatheca that affects ovulation and sperm competition (Mueller et al., 2008).

Results

First, I analyzed male sperm migration in uteri that lacked SWM-1. To do this, I bypassed the premature sperm activation defect of *swm-1* mutants by using *snf-10* mutants. SNF-10 is an SLC-6 transporter on the surface of the sperm cell membrane that was identified in a *swm-1* suppressor screen (Fenker et al., 2014). *snf-10* is downstream of *swm-1* and mutations in *snf-10* suppress the premature sperm activation of *swm-1* mutants. Importantly, while *snf-10* mutants cannot respond to TRY-5 in males, they are efficiently transferred to hermaphrodites and can activate *via* the hermaphrodite sperm activation pathway with no effect on male fertility (Fenker et al., 2014). Therefore, I analyzed sperm migration of *swm-1 snf-10* double mutants in the uteri of *swm-1* mutant hermaphrodites using males dyed with mitotracker, which labels mitochondria, allowing me to visualize sperm migration. I also included the temperature sensitive *fem-1(hc17ts)* mutation which renders hermaphrodites self-sterile because they do not produce self sperm when reared at the restrictive temperature (Doniach and Hodgkin, 1984). This allowed me to target to the analysis of the effect of male-derived SWM-1 on male fertility. I found that as compared to *snf-10* control sperm, *snf-10 swm-1* mutant sperm migration is normal (Figure A.1).

To test *swm-1* for a role in sperm competition, I set up matings with animals that lacked SWM-1 in the uterus and assessed sperm success by counting total progeny. To create this uterine environment that lacked SWM-1, I used *swm-1 snf-10* double mutant

males, which bypasses the sperm activation phenotype of *swm-1* mutants because *snf-10* mutant males cannot prematurely activate in response to TRY-5 in the male but they can be trans activated *via* the hermaphrodite pathway. Importantly, *swm-1 snf-10* mutant sperm display wild-type levels of fertility but whether SWM-1 in the uterus is important for sperm competition or some other aspect of sperm success is unknown (Fenker et al., 2014). To specifically test the role of SWM-1 on male sperm success, I mated males to *swm-1 fog-2* mutant hermaphrodites. The *fog-2* mutation is a temperature-sensitive mutation that renders hermaphrodites self-sterile when reared at 25ºC because they do not produce sperm self sperm (Schedl and Kimble, 1988). This allowed me to test the role of male-derived SWM-1 on male sperm success.

I used a sequential mating paradigm to compete the sperm of two males and used the GFP transgene, *mIs11* in one of the males to determine paternity of the most successful sperm, which was determined by total progeny. In control sequential matings with two wild-type males, there was no bias in paternity, as has previously been shown. By contrast, in matings where SWM-1 was provided by the second male but not the first male, first male sperm showed precedence. Furthermore, when SWM-1 was completely absent in the uterus, the second male showed strong paternal bias (Figure A.2A). This result was robust, as three independent repeats of this experiment showed the same result. However, in control matings where I reversed the order of the male that contained the GFP transgene, the result was not recapitulated (Figure A.2B).

Discussion

These results may indicate that SWM-1 could be important for the retention of position in the spermatheca. In matings where SWM-1 was absent from the uterus, sperm from the second male outcompeted sperm from the first male. Yet, when SWM-1 was provided by the second male but not the first, first male sperm took precedence, perhaps because seminal fluid spreads into the site of fertilization faster than sperm can migrate to it, allowing first male sperm to utilize the SWM-1 to retain their position. This phenomenon of factors being important for retention of position is known as defensive sperm competitive ability. However, these results cannot be confirmed without recapitulating the effect in the control experiment where the male that contains the *mIs11*. This control experiment however was only repeated once and perhaps warrants further testing. Going forward, further SWM-1 competition experiments may benefit from a more sensitive assay. Since SWM-1 derived from somatic body wall muscle is required for fertility but seminal fluid SWM-1 is not, perhaps its role as a seminal fluid is subtle and only provides a slight benefit to the reproductive process.

Materials and methods

Sperm competition assay

24 hour post L4 adult males were placed with 24 hour post L4 adult hermaphrodites and allowed to mate for 3 hours in a 1:1 ratio followed by removal of the first male and addition of the second male, which was also allowed to mate for 3 hours and then removed from the plate. Hermaphrodites were then transferred to fresh plates

and transferred every 16 hours. The mIs11 GFP transgene was included in the second male to determine paternity of offspring and total progeny were counted.

Sperm migration

Sperm migration was visualized and quantified by labeling males with the mitochondrial dye Mitotracker Red CMXRos (Life Technologies) as described in Stanfield and Villeneuve (2006). Mitotracker labeled males were allowed to mate with hermaphrodites for 30 minutes and then removed and mated hermaphrodites were analyzed to determine the position of sperm in the uterus.

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Figure A.1 swm-1 mutant sperm migration. Heat map showing the position of mitrotracker labeled sperm in the uterus in 30 minute intervals over a period of 3 hours. Position of sperm in each hermaphrodite is shown for wild-type (top rows, blue shading), *snf-10* (middle rows, green shading), and *snf-10 swm-1* (bottom rows, pink shading) mutant male sperm. Numbers indicate the percent of sperm at each position in the uterus. Shade of red coloring correlates with percent of sperm at each position. Positions in the uterus are represented in egg-lengths from vulva. Abbreviations indicate egg-length, EL and spermatheca, Sp.

	30 min						1 hour						1.5 hours						2 hours						2.5 hours						3 hours				
	WT n=15																																	
Plate	1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.
1	30	30	30	10	0		14	14	14	36	21		5	5	5	42	42		6	6	6	46	37		4	4	4	31	57		4	4	8	42	42
2	33	33	33	0	0		17	17	17	33	17		6	6	6	41	41		8	8	8	33	43		5	5	5	57	29		5	5	5	43	43
3	29	36	29	7	0		13	13	33	20	20		6	6	9	36	43		0	0	0	45	55		6	6	26	31	31		2	6	6	43	43
6	30	30	30	10	0		7	7	14	43	29		4	4	4	43	43		3	3	3	55	36		3	3	3	50	42		3	3	3	50	42
8	71	29	0	0	0		0	50	25	25	0		10	10	10	10	60		5	5	5	51	34		6	6	6	40	40		0	0	0	0	100
10	29	29	29	14	0		8	8	17	42	25		7	7	11	50	25		6	6	17	36	36		4	4	4	63	25		6	6	10	39	39
13	20	30	20	20	10		7	7	14	43	29		7	7	14	43	29		6	6	9	36	43		7	7	13	33	40		7	7	20	33	33
15	51	21	21	8	0		0	38	25	38	0		25	25	25	25	0		25	25	25	25	0		11	11	11	11	56		10	10	10	10	60
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22	23	23	23	23	8		15	15	15	31	23		11	11	14	29	36		6	6	6	37	46		6	6	6	37	46		7	7	11	34	42
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28	14	21	21	29	14		7	7	13	33	40		7	7	27	33	27		3	10	13	40	33		11	11	17	33	28		6	6	13	38	38
	snf-10 n=15																																	
Plate	1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Spthca.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.
1	25	25	25	13	13		13	13	19	31	25		8	8	8	37	37		8	8	8	33	43		0	5	5	36	54		3	3	3	53	37
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7	40	20	20	20	0		6	6	6	37	46		3	3	3	46	46		3	3	3	50	42		11	3	3	46	37		3	3	3	50	40
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16	33	33	17	17	0		13	13	13	13	50		13	13	13	13	50		0	0	0	40	60		8	6	6	40	40		8	8	8	8	67
17	25	25	25	25	0		18	36	9	18	18		8	8	8	51	26		4	4	4	38	51		7	11	19	19	44		5	5	5	29	57
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30	33	22	22	22	0		17	17	17	17	33		0	0	0	0	100		0	0	0	0	100		0	0	0	0	100		0	0	0	0	100
	snf-10 swm-1																																	
Plate	1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.		1EL	2EL	3EL	4 EL	Sp.
1	32	21	21	21	5		6	6	9	36	43		5	5	5	39	47		0	0	18	45	36		6	6	6	51	31		8	8	23	38	23
2	20	20	20	40	0		6	6	6	41	41		5	5	5	42	42		4	4	4	45	45		3	3	3	40	50		3	3	3	40	50
5	44	33	22	0	0		18	18	18	27	18		12	12	12	37	28		8	8	14	25	44		10	10	16	25	38		16	16	16	16	38
6	25	17	25	33	0		6	6	13	38	38		6	6	6	51	31		7	7	11	42	34		7	7	12	37	37		5	5	5	51	34
7	22	33	22	22	0		9	9	9	44	29		4	4	4	75	13		5	5	5	43	43		5	5	5	43	43		5	5	5	36	50
9	50	50	0	0	0		40	40	20	0	0		6	6	6	6	75		22	22	22	22	11		0	0	0	0	100		0	0	0	100	0
10	100	0	0	0	0		11	11	22	22	33		6	6	6	6	75		5	5	5	43	43		0	0	0	67	33		4	4	4	38	50
13	50	25	25	0	0		7	7	13	40	33		3	3	3	40	50		3	3	3	40	50		3	3	3	36	55		6	16	16	38	25
15	36	18	18	18	9		6	6	10	39	39		0	0	0	44	56		5	5	5	42	42		5	5	5	42	42		6	6	6	41	41
17	38	38	25	0	0		13	13	25	25	25		5	5	5	43	43		7	7	7	34	45		9	9	9	21	51		12	12	19	29	29
18	33	33	33	0	0		29	29	29	14	0		17	17	17	17	33		8	8	8	8	67		8	8	8	8	67		0	0	0	0	100
19	33	22	22	22	0		9	9	9	44	29		4	4	4	33	56		5	5	5	29	57		0	0	0	40	60		6	6	6	20	61
20	36	18	18	18	9		8	8	15	38	31		8	8	8	50	28		3	3	3	40	50		3	3	3	40	50		0	0	0	38	63
22	29	29	29	14	0		9	9	9	44	29		11	11	11	26	43		6	6	6	6	75		0	0	0	25	75		0	0	0	25	75
28	22	22	22	33	0		8	8	8	8	67		4	4	4	25	63		6	6	6	23	60		3	3	3	45	45		4	4	4	43	43
29	22	22	22	33	0		6	6	6	41	41		6	6	6	40	40		7	7	7	45	34		0	0	0	29	71		0	0	0	33	67

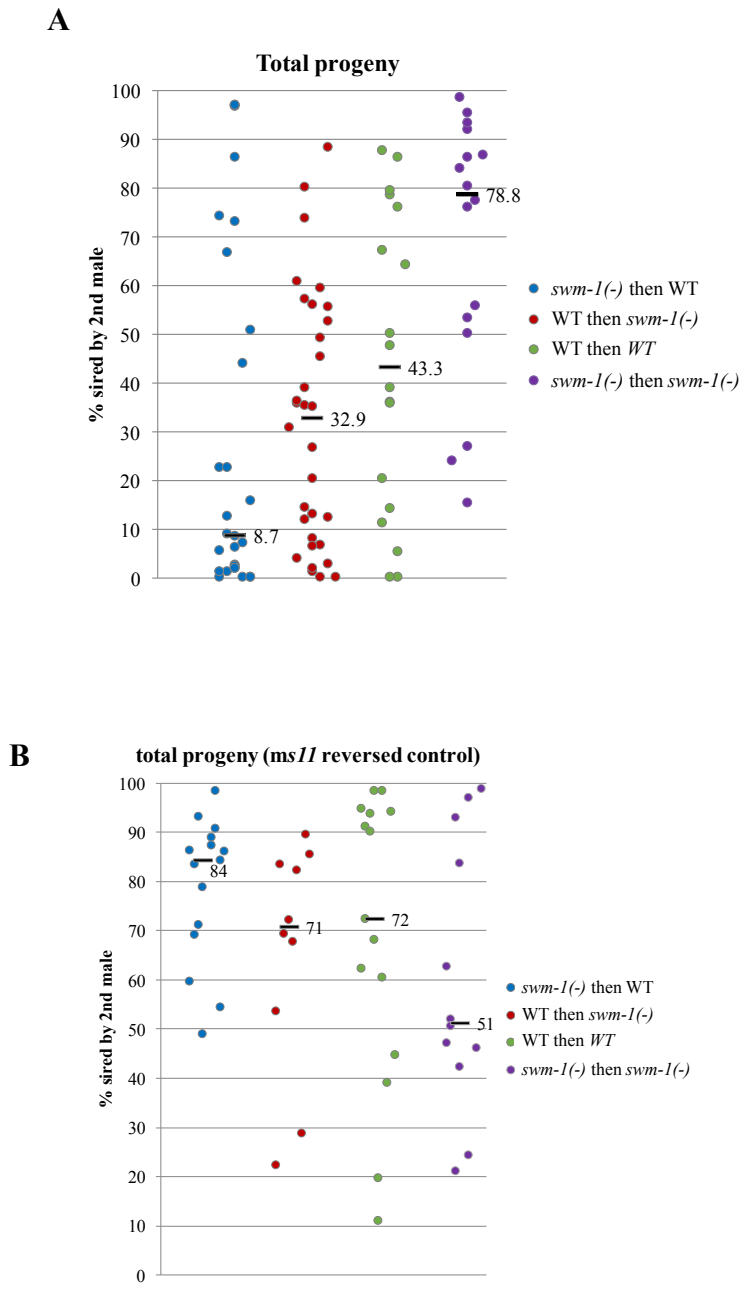

Figure A.2 Sperm competition in SWM-1 mutant males. A) Total progeny sired by the second male from sequential matings between wild type and *swm-1* mutant males. "second males" harbored the *mIs11* transgene. B) Control experiment with first males harboring the *mIs11* transgene.